

**CORTICAL GLUTAMIC ACID DECARBOXYLASE 67 EXPRESSION IN  
SCHIZOPHRENIA: DEFINING THE DEFICIT**

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University of Pittsburgh, 2011

Cognitive impairments are a core feature of schizophrenia and the best predictor of functional outcome, though current pharmacotherapies offer only limited cognitive improvement. Cognitive deficits span multiple domains and thus may reflect an overarching alteration in cognitive control, the ability to adjust thoughts or behaviors to achieve goals. Cognitive control depends on the dorsolateral prefrontal cortex (DLPFC), which exhibits altered activity in schizophrenia. DLPFC dysfunction is thought to be due, at least partially, to alterations in interneurons, which are regulated by levels of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD). A deficit in the 67 kDa isoform (GAD67), responsible for the majority of cortical GABA synthesis, has been widely replicated in the DLPFC of subjects with schizophrenia and is particularly prominent in the parvalbumin (PV)-containing subclass of interneurons. However, little is known about the relationship of DLPFC GAD67 mRNA levels and medication use, substance abuse, and illness severity and chronicity; translation of the transcript into protein; or protein levels in axon terminals, a key site of GABA production and function. Additionally, alterations in other GABA neurotransmission markers, including lower PV and GABA membrane transporter 1 (GAT1), are also present, and thought to result from lower GAD67 in PV neurons, though this hypothesis has not been directly tested. Accordingly, here we measured GAD67 mRNA, tissue-level protein, and axon terminal protein in PV cells, and examined whether lower PV and GAT1 mRNA are consequences of lower GAD67 protein in PV neurons. GAD67 mRNA levels were significantly 15% lower in schizophrenia subjects, but transcript levels were not associated with medication use, substance abuse, predictors or measures of disease severity, or illness chronicity. GAD67 protein levels were significantly 10% lower in total gray matter and 49% lower in PV axon terminals. These data provide an extensive



characterization of the GAD67 deficit in schizophrenia, and provide novel evidence of a functional impairment in PV neurons that may underlie cognitive deficits. Additionally, PV and GAT1 mRNAs were not altered in two mouse models with lower GAD67 expression, suggesting that lower GAD67 is unlikely to be the cause of reduced PV and GAT1 mRNA in schizophrenia.

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## LIST OF ABBREVIATIONS

AIS: axon initial segment  
ANOVA: analysis of variance  
ANCOVA: analysis of covariance  
ATOD: at time of death  
BDNF: brain derived neurotrophic factor  
CB1: cannabinoid 1 receptor  
CCK: cholecystokinin  
Cdc42: cell division cycle 42  
CR: calretinin  
CV: coefficient of variation  
DLPFC: dorsolateral prefrontal cortex  
DSM-IV-TR: *Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition, Text Revision*  
 $E_{\text{GABA}}$ : GABA reversal potential  
E/I: excitatory/inhibitory  
EPSC: excitatory postsynaptic current  
FS: fast-spiking  
GABA:  $\gamma$ -aminobutyric acid  
GAD: glutamic acid decarboxylase  
GAD65: 65 kDa isoform of glutamic acid decarboxylase  
GAD67: 67 kDa of glutamic acid decarboxylase  
GAPDH: glyceraldehyde-3-phosphate dehydrogenase  
GAT1: GABA membrane transporter 1  
IPSC: inhibitory postsynaptic current

IPSP: inhibitory postsynaptic potential  
ISH: *in situ* hybridization  
IR: immunoreactive  
KCC2: potassium/chloride cotransporter 2  
M1: primary motor cortex  
M2: secondary motor cortex  
MCID: Microcomputer Imaging Device  
MD: medial dorsal  
mPFC: medial prefrontal cortex  
nCi/g: nanoCuries per gram  
non FS: non fast-spiking  
NR1: NMDA receptor 1  
kDa: kilodalton  
KO: knockout  
OD: optical density  
P: postnatal day  
PFC: prefrontal cortex  
PMI: postmortem interval  
PV: parvalbumin  
PV<sub>b</sub>: parvalbumin basket  
PV<sub>ch</sub>: parvalbumin chandelier  
qPCR: quantitative polymerase chain reaction  
rCBF: regional cerebral blood flow  
RIN: RNA integrity number  
SD: standard deviation  
SES: socioeconomic status  
SNP: single nucleotide polymorphism  
SST: somatostatin  
TrkB: tyrosine kinase B  
VIP: vasoactive intestinal peptide  
V<sub>rest</sub>: resting membrane potential

For my uncle,  
Craig Allen Kerr,

Whose battle with schizophrenia is over, but not forgotten

## PREFACE

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## **1.0 GENERAL INTRODUCTION**

### **1.1 OVERVIEW OF SCHIZOPHRENIA**

#### **1.1.1 Burden of schizophrenia**

Schizophrenia is a devastating neuropsychiatric illness that affects approximately 0.5-1% of the population worldwide (Lewis and Lieberman, 2000). Symptoms generally appear in late adolescence or early adulthood, and most affected individuals experience a life-long course of illness characterized by difficulties with employment, personal relationships, and self-care. Schizophrenia is the 14<sup>th</sup> leading cause of disability worldwide and accounts for 3% of the total of years of healthy life lost due to disability (*Global Burden of Disease: 2004 Update*, 2008). In fact, approximately 30% of schizophrenia patients have attempted suicide (Radomsky et al., 1999), and around 5% will eventually die from it (Hor and Taylor, 2010; Palmer et al., 2005).

In addition to the personal suffering experienced by patients themselves, schizophrenia also places a substantial burden on caregivers as well as society. Family members are often the primary caretakers, and as such experience a significant emotional as well as financial load (Gibbons et al., 1984). Schizophrenia is also very costly to society as a whole. In 2002, schizophrenia cost the U.S. an estimated \$62.7 billion dollars (Wu et al., 2005). This figure includes \$22.7 billion in direct health care costs as a result of pharmacological treatment and inpatient hospitalizations, as well as \$32.4 billion in indirect costs of loss of productivity due to unemployment and premature mortality. In fact, reports from the U.S. and European countries estimate that the cost of schizophrenia is 1-3% of the total national healthcare expenditure (Knapp et al., 2004).

### 1.1.2 Clinical features of schizophrenia

Symptoms of schizophrenia fall principally into 3 categories: positive, negative, and cognitive. Positive symptoms, present in addition to or as a distortion of normal behavior, are broadly characterized by an altered perception of reality, and are generally the features of schizophrenia that first bring an individual to clinical attention. Positive symptoms include delusions, hallucinations, disorganized thought and behavior, and catatonia. Delusions, false beliefs that persist in the face of contrary evidence, typically include one or more themes (e.g. persecution, reference, religion, or grandeur). The most common forms are delusions of persecution, where the patient believes he or she is being harassed or tormented, and delusions of reference, in which the patient believes certain environmental cues, such as newspaper headlines, are being purposely directed at him or her. Hallucinations, sensory perceptions in the absence of external stimuli, can occur in any sensory modality, but most often are auditory, with the patient hearing voices (often two or more in conversation with each other) that are distinct from their own thoughts. Disorganized thought describes language deficits that are assumed to result from impairments in thought, and focuses on speech problems (disorganized speech) that can be easily assessed in a clinical setting. Disorganized speech can manifest in a variety of ways, such as difficulty maintaining a single topic, providing unrelated answers in response to a question, and even total incoherence known as “word salad.” Disorganized behavior is also similarly heterogeneous, and may include disheveled appearance, inappropriate sexual behavior, or unpredictable agitation or silliness. Finally, catatonia, a decreased or totally absent response to the surrounding environment, can range from rigid posturing, to resistance to movement, to total unawareness [*Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR)*, 2000].

Conversely, negative symptoms represent a loss of behavior generally present in normal individuals, and result in impaired emotional expression and social functioning. The three main negative symptoms are flattened affect, alogia, and avolition, though others such as anhedonia may also be present. Flattened affect is the most common, and results in the patient exhibiting a reduced range of emotional expressions, as well

as diminished eye contact and body language. Alogia, or poverty of speech, is characterized by laconic and empty verbal responses. Avolition, or lack of motivation, results in difficulty initiating and pursuing actions and consequently little or no engagement in work or social activity. Anhedonia is an inability to take pleasure from activities that used to be enjoyable (DSM-IV-TR, 2000).

Currently, the DSM-IV-TR (2000) diagnostic criteria of schizophrenia are based solely on clinical symptoms and a diagnosis is made following a behavioral disturbance lasting at least 6 months. During this time, the patient must exhibit impaired social and/or occupational functioning (such as work, interpersonal relationships, or self-care) for a majority of the time, as well as the presence of two or more of the following acute symptom classes during the majority of a 1-month period: delusions, hallucinations, disorganized speech, disorganized or catatonic behavior, and negative symptoms.

Finally, cognitive impairments, not presently included in the diagnostic criteria, but featured prominently in the DSM-IV-TR (2000) description of the illness, are thought to represent a core feature of schizophrenia (Elvevag and Goldberg, 2000), which will be explored in more detail below. The National Institute of Mental Health has recognized the need for better treatment of cognitive symptoms in schizophrenia, and, to that end, has formed several initiatives including Cognitive Neuroscience Treatment to Improve Cognition in Schizophrenia (CNTRICS) (Carter and Barch, 2007). CNTRICS has identified six cognitive domains that are altered in schizophrenia: working memory, executive function, episodic memory, attention, perception, and social/emotional processing (Carter et al., 2008).

### **1.1.3 Etiology of schizophrenia**

Substantial evidence suggests that genetics play a strong role in schizophrenia, with current heritability estimates around 80% (reviewed in Cardno and Gottesman, 2000). In contrast to the 1% risk of developing schizophrenia in the general population, relatives of schizophrenia patients have an increased risk of developing the disorder, and this risk is directly proportional to genetic relatedness to the affected individual (Gottesman, 1991). Data pooled from 40 studies of twin pairs conducted between 1920 and 1987 in

western Europe demonstrate that the lifetime risk of developing schizophrenia is 17% for the unaffected twin of a dizygotic pair, but 48% for the unaffected twin of a monozygotic pair (Gottesman, 1991), who share 50% and 100% of their DNA with the affected twin, respectively. Additionally, an elevated risk of developing schizophrenia is present in both the adopted-away biological offspring of schizophrenia patients (Rosenthal et al., 1971), as well as offspring of monozygotic twins discordant for schizophrenia, regardless of whether the parent was the affected or unaffected twin (Gottesman and Bertelsen, 1989). In fact, numerous studies have demonstrated that many regions of the genome, as well as a plethora of individual genes, are associated with schizophrenia, (reviewed in Owen et al., 2004; Gejman et al., 2010). To date, however, the effects of the identified variants have been disappointingly small, and unable to account for such high heritability of the illness (Insel, 2010).

In addition to highlighting the role of genetics in schizophrenia, twin studies also point to a substantial role for the environment in the etiology of schizophrenia. If genetic liability was the sole pathogenic feature of schizophrenia, then monozygotic twin pairs would be 100% concordant for the presence or absence of the illness, which, as reviewed above, is not the case. Additionally, some 60% of individuals with schizophrenia do not have a first or second degree relative with the illness (Gottesman, 1991). This suggests that environmental factors must also play a role. In fact, several environmental risk factors, ranging throughout development, have been identified. Early developmental risk factors include advanced paternal age (Sipos et al., 2004), winter or spring birth season (Torrey et al., 1997; Davies et al., 2003), abnormal fetal growth and development, complications of pregnancy, and complications of delivery (Cannon et al., 2002a). Risk factors later in development include an urban upbringing versus a rural one (McGrath et al., 2004; March et al., 2008), migrant as opposed to native-born individuals (Bourque et al., 2011; McGrath et al., 2004; Cantor-Graae and Selten, 2005), childhood trauma (Read et al., 2005; Schreier et al., 2009), and cannabis use during adolescence (Zammit et al., 2002; Minozzi et al., 2010).

Since neither genes nor environment can explain schizophrenia alone, current etiological models of schizophrenia posit the combined role of multiple genetic and environmental “hits” (Bayer et al., 1999; Maynard et al., 2001). Based on the idea that

many risk factors occur during early development, long before symptom onset, this neurodevelopmental hypothesis of schizophrenia suggests that combined genetic and environmental risk factors affect normal developmental processes to produce disease onset much later during the protracted maturation that occurs during adolescence and early adulthood (Weinberger, 1987; Insel, 2010).

#### **1.1.4 Cognitive symptoms are a central feature of schizophrenia**

In 1887, Dr. Emile Kraepelin first described a group of symptoms he called “dementia praecox” (Kraepelin, 1919), which was later termed “schizophrenia” by Eugene Bleuler in 1911 (Bleuler, 1950). Although cognitive impairments were prominently featured in Kraepelin’s description of the illness, modern diagnostic criteria do not include cognitive symptoms (DSM-IV-TR, 2000). Recently, however, the cognitive aspects of schizophrenia have received increased attention, and are now thought to represent a core feature of schizophrenia for a number of reasons (reviewed in Elvevag and Goldberg, 2000; Gold, 2004). First, a large body of literature has replicated broad and severe cognitive deficits in schizophrenia, with nearly all cognitive domains affected to some degree by the illness (Heinrichs and Zakzanis, 1998; Nuechterlein et al., 2004) and patients performing, on average, 1-2 standard deviations below the mean of comparison subjects (Heaton et al., 2001; Wilk et al., 2004). Second, cognitive impairment in schizophrenia is widespread, and the majority (70-80%) of patients perform worse than the average of comparison subjects on neuropsychological tests (Palmer et al., 1997; Wilk et al., 2004). In fact, cognitive impairments may actually be even more prevalent than these data suggest, since even patients who score within the normal range of these tests do exhibit some form of cognitive dysfunction (Allen et al., 2003; Kremen et al., 2000). Additionally, when cognitive dysfunction is defined as falling below predicted levels (based on premorbid intellectual functioning and parental education), 98% of schizophrenia patients are impaired (Keefe et al., 2005). Moreover, in studies of monozygotic twins discordant for schizophrenia, the ill twin performed worse than the healthy twin in nearly all cases (Goldberg et al., 1990; Goldberg et al., 1995).

Cognitive deficits appear to be a primary factor of the illness. Treatment with antipsychotics provides little to no relief from cognitive symptoms (Goldberg et al., 1993; Gold, 2004; Meltzer and McGurk, 1999; Harvey and Keefe, 2001) and the severity of positive symptoms is not related to the severity of cognitive impairment (Heydebrand et al., 2004), suggesting that cognitive impairments are not secondary to the psychotic symptoms. In fact, cognitive impairments are present long before illness onset in children (Reichenberg et al., 2010; Niendam et al., 2003) and adolescents (Davidson et al., 1999; Cornblatt et al., 1999) who later go on to develop schizophrenia, and therefore may represent a risk factor for later developing schizophrenia. Unaffected relatives of patients exhibit similar, though milder, cognitive impairments (Sitskoorn et al., 2004; Egan et al., 2001), which suggests a genetic component to these symptoms. Additionally, the severity and pattern of cognitive deficits is stable across the course of the illness (Aleman et al., 1999; Censits et al., 1997) and deficits are present in medication-naïve, first-episode patients (Mohamed et al., 1999; Saykin et al., 1994), suggesting that cognitive impairment is not a consequence of treatment, length of illness, or hospitalization.

Finally, cognitive impairments are the best predictor of long-term functional outcome (reviewed in Green et al., 2000), better than both positive and negative symptoms. Studies that utilize composite measures of cognition (Velligan et al., 1997; Harvey et al., 1998; Bartels et al., 1997), as well as those that have examined specific cognitive domains (Addington and Addington, 1999; Lysaker et al., 1995), have demonstrated that cognitive ability predicts social and occupational functioning, self-care, and quality of life. For example, a recent meta-analysis that examined 62 studies published since 1998 found that cognitive functioning, but not severity of positive symptoms, was a significant predictor of employment outcome (Tsang et al., 2010).

### **1.1.5 Treatment and outcome in schizophrenia**

Since their introduction in the 1950s, antipsychotic drugs have been the mainstay of pharmacological treatment for schizophrenia. Early drugs, such as chlorpromazine, belong to the class of “typical” antipsychotics, and act through a blockade of dopamine

D2 receptors. The more recently developed “atypical” antipsychotics, such as clozapine and olanzapine, are associated with fewer side effects and block serotonin as well as dopamine receptors. In general, both generations of antipsychotics reduce the positive symptoms of the illness, but have consistently been shown to have little to no effect on the negative (Buchanan et al., 1998) and cognitive symptoms of schizophrenia (Goldberg et al., 1993; Harvey and Keefe, 2001). Despite the widespread use of antipsychotics today, outcome in schizophrenia is still bleak (Insel, 2010). Five years after a first psychotic episode, only 14% of patients have experienced a prolonged recovery period (Robinson et al., 2004), and only an additional 16% are in recovery 15 years later (Harrison et al., 2001).

A number of epidemiological factors predict the severity of schizophrenia symptoms. For example, there is a dramatic sex effect in the illness. Relative to females, males have a higher incidence of schizophrenia (McGrath et al., 2004; Aleman et al., 2003), an earlier onset of symptoms (Shimizu et al., 1988; Castle et al., 1998), and a more severe illness course (Goldstein, 1988; Grossman et al., 2008). Additionally, a diagnosis of “pure” schizophrenia, as opposed to schizoaffective disorder, which is defined by the addition of significant mood symptoms, is also associated with greater positive, negative, and cognitive symptom severity (Cheniaux et al., 2008). A positive family history of schizophrenia is a third predictive factor of both more severe negative symptoms (Malaspina et al., 2000) and cognitive dysfunction (Tabarés-Seisdedos et al., 2003). Finally, a lower age of onset (< 18 years) is also associated with a more severe illness course (Werry et al., 1994; Hollis, 2000).

In addition to factors that predict more severe schizophrenia symptomatology, a number of general life benchmarks, including history of marriage, employment, educational achievement, and independent living, can be used as indices of functional outcome (Figueira and Brissos, 2011; Jönsson and Jonsson, 1992; Thara and Srinivasan, 1997). In fact, it is somewhat obvious that patients who are able to fulfill societal roles of spouse, employee, student, and friend are less functionally impaired. Since antipsychotics have little to no effect on cognitive symptoms (Goldberg et al., 1993), the best predictor of functional outcome (Green et al., 2000), it is not surprising that antipsychotic treatment has not significantly improved functional outcome in the

illness (Insel, 2010). For example, recent employment rates among European schizophrenia subjects are below 20% (Marwaha et al., 2007), and nearly 20% of U.S. patients are homeless (Folsom et al., 2005). Thus, treatments to ameliorate cognitive deficits and consequently improve functional outcome in schizophrenia are of paramount importance.

## **1.2 IMPAIRMENTS IN COGNITIVE CONTROL: ROLE OF DORSOLATERAL PREFRONTAL CORTICAL GABA NEURONS**

Cognitive impairments in schizophrenia are a core feature of the illness (Elvevag and Goldberg, 2000), have the most severe impact on patient quality of life (Hyman and Fenton, 2003), and are the best predictor of functional outcome (Green et al., 2000). Unfortunately, current treatments for schizophrenia have little effect on cognitive symptoms (Gold, 2004). Central to the development of new treatment targets for cognitive deficits is an understanding of the underlying abnormalities in the brain regions, circuits, and cells that are affected in the illness.

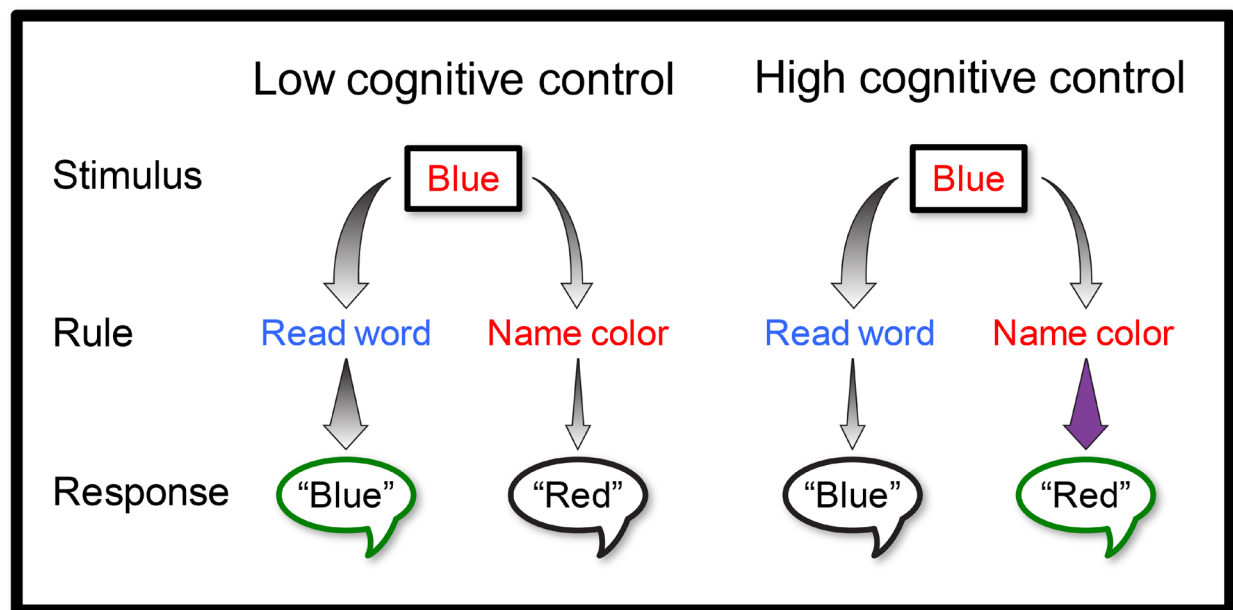
### **1.2.1 Cognitive impairments in schizophrenia may reflect an overarching deficit in cognitive control**

As reviewed above, schizophrenia is characterized by a broad pattern of cognitive deficits across a variety of domains. One hypothesis is that these deficits reflect distinct alterations in multiple cognitive systems; however, since a single neuropsychological test typically activates multiple cognitive processes, it is difficult to determine the affected domains (Dickinson and Gold, 2008). Alternatively, a more recent and parsimonious idea suggests that impaired cognition in schizophrenia may reflect an overarching dysfunction in cognitive control, the ability to adjust thoughts or behaviors in order to achieve goals, which manifests as deficits in multiple cognitive domains (reviewed in Lesh et al., 2011). In fact, this view was shared by Kraepelin, who, in his



original account of the illness, described the schizophrenia brain quite elegantly as an “orchestra without a conductor” (Kraepelin, 1919).

A simple illustration of cognitive control is observed in the classic measure of directed attention, the Stroop task (Stroop, 1935), in which the subject is presented with various color words (red, blue, green, etc.) written in an ink color that is either congruent or incongruent with the word (e.g. the word “blue” written in blue ink, and the word “blue” written in red ink, respectively). In the low cognitive control condition, the subject must read the word, ignoring the ink color. In the more difficult, higher cognitive control condition, the patient must ignore the prepotent word-reading response and instead name the ink color (**Figure 1**). When presented with conflicting behavioral choices, this flexible adaptation that allows for the appropriate response selection is the essence of cognitive control (Lesh et al., 2011). Schizophrenia subjects consistently perform worse than healthy comparison subjects on the Stroop task, exhibiting longer reaction times and an increased number of errors (Breton et al., 2011; Cohen et al., 1999).



**Figure 1. Cognitive control illustrated using the Stroop task.** Subjects must either read the word or name the color of ink the word is printed in. The correct responses for each condition are outlined in green. In the low cognitive control condition, the subject is

easily able to give the predominant response and read the word, correctly answering “blue”. In the more difficult, high cognitive control condition, the subject must ignore the prepotent word-reading tendency, and instead name the word color, “red”. Adapted by permission from Macmillan Publishers Ltd: Neuropsychopharmacology (Lesh et al., 36:316-18), copyright 2011.

In fact, cognitive control involves multiple cognitive processes besides attention, including the well-studied domain of working memory (Lesh et al., 2011). Schizophrenia subjects exhibit deficits on a variety of tasks that engage cognitive control, including those that involve working memory (Fleming et al., 1995; Gold et al., 1997; Goldberg et al., 1998; Perlstein et al., 2001). Working memory, the active maintenance of a small amount of information for a short period of time in order to influence future thought or behavior, is thought to be composed of four components: 1) an articulatory loop that maintains and rehearses verbal information, 2) a visuospatial scratchpad that maintains visual information, 3) an episodic buffer that stores information integrated from multiple modalities and 4) a central executive system that manipulates and controls information within the first three storage buffers (Baddeley, 1992; Baddeley, 2000). Consistent with an overarching deficit in cognitive control, subjects with schizophrenia are consistently impaired on measures of the central executive component (reviewed in Barch, 2006; Kim et al., 2004).

### **1.2.2 Role of the DLPFC in cognitive control deficits in schizophrenia**

Cognitive control depends on a number of brain regions, including dorsolateral prefrontal cortex (**DLPFC**), anterior cingulate cortex, and parietal cortex (Braver et al., 1997; Cohen et al., 1997; Pardo et al., 1990; Paus et al., 1993; Bench et al., 1993; Braver et al., 2003). Based on its widespread anatomical connections with a variety of other brain regions, including sensory, motor, and intrinsic connections to other

prefrontal cortex (**PFC**) regions, the PFC is thought to play an especially central role in cognitive control (reviewed in Miller and Cohen, 2001). The PFC is critical for top-down control of behavior, and through the integration of extrinsic and intrinsic information from other brain areas, its neural activity represents the rules required to perform a given task (Asaad et al., 1998; White and Wise, 1999; Petrides, 1990). Specifically, the DLPFC is thought to be critically involved in maintenance of rules and selection of the appropriate response (Asaad et al., 2000; Watanabe, 1990; Watanabe, 1992).

Impairments in DLPFC functioning in schizophrenia are well-established (reviewed in Weinberger, 2001). The first evidence came from a regional cerebral blood flow (**rCBF**) study, reflective of neuronal activity, demonstrating hypofrontality in schizophrenia (Ingvar and Franzén, 1974). About a decade later, a series of seminal rCBF studies found reduced DLPFC activation at rest and during a cognitive control task, the Wisconsin Card Sort, in both medication-free (Weinberger et al., 1986; Weinberger et al., 1988) and antipsychotic-treated (Berman et al., 1986) schizophrenia subjects. Additionally, DLPFC rCBF was significantly correlated with performance on the Wisconsin Card Sort (Weinberger et al., 1986). An abundance of functional magnetic resonance imaging (fMRI) studies have since examined DLPFC function during cognitive control tasks. Consistent with the rCBF data, the majority of studies have reported DLPFC hypoactivity (Minzenberg et al., 2009; Glahn et al., 2005; Taylor, 1996; Andreasen et al., 1992; Perlstein et al., 2001), though others have demonstrated hyperactivity (Callicott et al., 2000; Manoach et al., 2000; Manoach et al., 1999). It seems that subjects with schizophrenia exhibit a relationship between working memory load, behavioral performance, and DLPFC activation that is more complex than a simple decrease or increase in function. It has been demonstrated that healthy subjects exhibit an “inverted U”-shaped relationship between DLPFC activation and working memory load, exhibiting lower activation levels at both low loads and high loads, and higher activation at intermediate loads (Callicott et al., 1999). In schizophrenia subjects, this inverted U is thought to be shifted to the left, so that schizophrenia subjects exhibit greater DLPFC activation and have relatively normal performance at low working memory loads, but reach the peak of the inverted U sooner, resulting in reduced activation and impaired performance at higher loads (Callicott et al., 2003; Van

Snellenberg et al., 2006; Potkin et al., 2009; Manoach, 2003). Additionally, affected monozygotic twins exhibit reduced gray matter volume in the DLPFC compared to their unaffected twin, and these reductions correlate with the degree of cognitive impairment (Cannon et al., 2002b).

### **1.2.3 DLPFC $\gamma$ oscillations underlie cognitive control and are impaired in schizophrenia**

Oscillations in the  $\gamma$  band, the synchronized activity of networks of pyramidal neurons at 30-80 Hz, are thought to underlie higher order cognitive processes (Fries, 2009). Studies in humans have shown that  $\gamma$  band activity is induced during the delay period of working memory tasks (Tallon-Baudry et al., 1998), and that the power, or amplitude, of  $\gamma$  synchrony increases in proportion to working memory load (Howard et al., 2003). However, this increase in  $\gamma$  band power is absent in subjects with schizophrenia (Cho et al., 2006; Haenschel et al., 2009; Minzenberg et al., 2010). Additionally, the phase-locking of  $\gamma$  oscillations at the onset of a stimulus during a working memory task is impaired in schizophrenia patients (Spencer et al., 2003). Importantly, first-episode, medication-naïve patients exhibit  $\gamma$  oscillation impairments (Minzenberg et al., 2010), indicating that they are not likely a consequence of illness chronicity or medication use. Therefore, the impairments in cognitive control that are observed in schizophrenia may be due to impaired  $\gamma$  oscillations in the DLPFC.

### **1.2.4 Role of DLPFC interneurons in $\gamma$ oscillations and cognitive control**

Oscillations are dependent on signaling through interneurons that signal via the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (**GABA**) (Bartos et al., 2007; Whittington et al., 1995). A single interneuron not only makes multiple contacts onto a pyramidal cell, but also contacts several different pyramidal cells (Gonzalez-Burgos and Lewis, 2008). This places interneurons in a position to exert strong inhibitory control over large numbers of pyramidal cells, so that firing of an interneuron transiently silences many pyramidal cells. When the pyramidal cells are then released from inhibition, they fire in

concert (Gonzalez-Burgos and Lewis, 2008). Thus, inhibitory postsynaptic potentials (**IPSPs**) arising from a single GABA neuron are thought to be capable of synchronizing its postsynaptic target cells (Cobb et al., 1995). Furthermore, interneurons are connected to other interneurons by both chemical and electrical synapses (gap junctions) (Mann and Paulsen, 2007), and modeling studies have demonstrated that connected interneurons are a mechanism of synchrony (Van Vreeswijk et al., 1994). Different subtypes of GABA interneurons appear to play distinct roles in the generation of oscillations of different frequencies (reviewed in Klausberger and Somogyi, 2008), and the parvalbumin (**PV**)-expressing subclass of interneurons are especially crucial in  $\gamma$  oscillations (reviewed in Gonzalez-Burgos, 2010).

### 1.3 IMPAIRMENTS IN DLPFC INTERNEURONS IN SCHIZOPHRENIA

Cognitive control and normal  $\gamma$  oscillatory activity relies on the proper functioning of DLPFC GABA neurons. Disturbances in GABA neurotransmission, then, are a plausible mechanism underlying cognitive control and  $\gamma$  oscillatory deficits in schizophrenia. Given the divergent roles of interneuron subtypes in DLPFC function, knowledge of the affected cells and molecules involved in schizophrenia will inform future treatment targets.

#### 1.3.1 GABA synthesis is consistently impaired in schizophrenia

Early findings of decreased activity (Bird et al., 1979; Sherman et al., 1991) of glutamic acid decarboxylase (**GAD**), the enzyme that produces GABA, and reduced GABA release (Sherman et al., 1991) and uptake (Simpson et al., 1989) suggested that cortical GABA signaling is lower in schizophrenia. The most consistent evidence in favor of this hypothesis are reports of a deficit in the mRNA encoding the 67 kilodalton (**kDa**) isoform of GAD (**GAD67**), the isoform responsible for the majority of GABA synthesis in the cortex, in the DLPFC of subjects with schizophrenia (reviewed in Gonzalez-Burgos,

2010). First demonstrated by Akbarian and colleagues using *in situ* hybridization (**ISH**) (1995b), lower GAD67 mRNA in the DLPFC has since been widely replicated using DNA microarray, quantitative PCR (**qPCR**), and ISH (Volk et al., 2000; Guidotti et al., 2000; Mirnics et al., 2000; Vawter et al., 2002; Hashimoto et al., 2005; Straub et al., 2007; Huang et al., 2007; Huang and Akbarian, 2007; Hashimoto et al., 2008b; Hashimoto et al., 2008a; Woo et al., 2008; Duncan et al., 2010).

On a cellular level, the density of GAD67 mRNA+ neurons is reduced by 25-35% across DLPFC layers I-V of subjects with schizophrenia (Akbarian et al., 1995b; Volk et al., 2000; Woo et al., 2008). However, in the remaining GAD67+ neurons, the expression level per neuron is not different from that of comparison subjects (Volk et al., 2000). One interpretation of these data is that a portion of GAD67 mRNA+ neurons are lost in schizophrenia. However, in light of several studies demonstrating no changes in total neuron number in the PFC in schizophrenia (Akbarian et al., 1995b; Thune et al., 2001), a more plausible hypothesis is that most GABA neurons in schizophrenia express normal levels of GAD67 mRNA, and a subset express the transcript at levels below detectability.

Although the decrement in GAD67 mRNA is one of the best-replicated findings in postmortem schizophrenia research, small sample sizes and often overlapping subject cohorts (in which different studies utilize a portion of the same subjects) point to the need for a large-scale study. Additionally, across the literature, substantial heterogeneity in levels of GAD67 are present within a cohort: most subjects with schizophrenia have lower GAD67 mRNA levels, but some subjects exhibit normal or even increased levels (Volk et al., 2000; Hashimoto et al., 2005; Hashimoto et al., 2008b). Just how common the GAD67 mRNA deficit is, as well as the factors that may be associated with the heterogeneity, is unknown. For example, although lower GAD67 is thought to underlie the cognitive deficits of the illness (Lewis et al., 2005), which are known to be the best predictors of functional outcome (Green et al., 2000; see **1.1.4**), the relationship between GAD67 mRNA levels and indices of functional outcome has not yet been examined. Furthermore, since GAD67 expression is activity-dependent (Benson et al., 1994), lower expression might just index the less stimulating social, occupational and intellectual environment associated with illness chronicity. Therefore,

further research is needed to address the heterogeneity of the deficit and examine the relationship between GAD67 mRNA, functional outcome, and length of illness.

Furthermore, despite the widely-replicated mRNA finding, GAD67 protein levels in the DLPFC have only been measured in a few studies, with both decreased (Guidotti et al., 2000) and unchanged (Dracheva et al., 2004; Straub et al., 2007) levels reported. Knowledge of protein levels is of particular importance given that mRNA and protein levels are not necessarily correlated, since a number of factors regulate transcription and translation (Nelson and Keller, 2007). For example, pharmacological manipulation of GABA levels is associated with changes in GAD67 protein but not mRNA (Rimvall et al., 1993). Additionally, GAD67 protein levels at the axon terminal, the principal site of GABA production and function, are unknown. Thus, studies are needed to establish GAD67 protein levels in schizophrenia.

In contrast to the reductions in GAD67, the mRNA and protein levels of the 65 kDa isoform of GAD (**GAD65**), the other enzyme responsible for GABA synthesis, are reportedly unaltered (Guidotti et al., 2000; Hashimoto et al., 2008a) or only slightly reduced (Hashimoto et al., 2008b) in the DLPFC in schizophrenia. The density of GAD65-immunoreactive (**-IR**) terminals is also unchanged (Benes et al., 2000). Thus, schizophrenia is characterized by a selective deficit in GAD67, but not GAD65, in the DLPFC.

The two GAD enzymes decarboxylate glutamate to form GABA and, together, GAD65 and GAD67 account for virtually all GABA synthesis in the cortex, although they each appear to have distinct roles. They are encoded by separate genes on different chromosomes (Erlander et al., 1991), subject to different post-translational modifications (Christgau et al., 1992), and have distinct intracellular distributions, although most GABA neurons appear to contain both isoforms (Hendrickson et al., 1994; Esclapez et al., 1994). GAD65 is primarily located at axon terminals, while GAD67 appears to be distributed throughout the neuron (Kaufman et al., 1991; Esclapez et al., 1994). Both GAD65 and GAD67 are subject to regulation by the cofactor pyridoxal 5'-phosphate, which activates and inactivates the enzyme when it is bound and released, respectively. GAD65 exists largely in the inactive form, with low levels of cofactor binding, and is highly regulated, while most GAD67 is saturated with cofactor and therefore in the

active form (Kaufman et al., 1991). Unlike GAD65, GAD67 is thought to be responsible for the majority of GABA synthesis in the cortex (Battaglioli et al., 2003; Mason et al., 2001). Elimination of the GAD65 gene in mice does not change levels of GABA in the cortex (Asada et al., 1996), while reducing GAD67 results in reduced GAD activity and GABA content (Asada et al., 1997). GAD65, on the other hand, seems to be mainly active during conditions of high synaptic demand (Battaglioli et al., 2003; Patel et al., 2006). Finally, GAD67 seems to be more sensitive to perturbation of the GABA system than GAD65 (Soghomonian and Martin, 1998), which is consistent with the selective reduction of GAD67 but not GAD65 in schizophrenia. Therefore, the reductions in GAD67 that are observed in the DLPFC of subjects with schizophrenia are thought to result in a reduction of cortical GABA levels that significantly impairs synaptic transmission and inhibition of their postsynaptic targets.

### **1.3.2 Deficits in other aspects of GABA neurotransmission are also present in schizophrenia**

Findings suggest that deficits in GABA reuptake are also present in the DLPFC of subjects with schizophrenia. Expression of the mRNA for the GABA membrane transporter 1 (**GAT1**), which removes GABA that has been released into the synapse, is decreased in the DLPFC of schizophrenia subjects (Ohnuma et al., 1999; Hashimoto et al., 2008a; Hashimoto et al., 2008b). There is also a lower density of GAT1 mRNA+ neurons (Volk et al., 2001). The deficits in GAD67 and GAT1 may be occurring in the same cells, since, in the same cohort of subjects, the pairwise differences in GAD67 and GAT1 mRNA+ neuron density were similar in laminar pattern and significantly correlated (Volk et al., 2001). Lower density of GAT1-IR structures (Woo et al., 1998; Pierri et al., 1999) and reduced GAT1 binding (Schleimer et al., 2004) has also been reported. These findings suggest that, in schizophrenia, there is a reduction in both the synthesis and reuptake of cortical GABA in a subpopulation of interneurons.

Alterations in postsynaptic ionotropic GABA<sub>A</sub> receptors are also present in schizophrenia. The GABA<sub>A</sub> receptor is composed of 5 subunits that form a central ion pore, with the most common composition being two  $\alpha$ , two  $\beta$ , and one  $\gamma$  subunit (Tretter



et al., 1997). Activation of these receptors results in an increased chloride conductance, inward chloride ion flow, and hyperpolarizing IPSPs. Lower mRNA levels of  $\alpha 1$  (Akbarian et al., 1995a; Hashimoto et al., 2008b; Hashimoto et al., 2008a; Beneyto and Lewis, 2010; but see Duncan et al., 2010),  $\beta 2$  (Beneyto et al., 2011), and  $\gamma 2$  (Akbarian et al., 1995a; Hashimoto et al., 2008a) subunits, which coassemble together in more than 60% of GABA<sub>A</sub> receptors in the cortex (Möhler, 2006), have been reported in the DLPFC. The lower  $\alpha 1$  mRNA levels are present on pyramidal neurons but not interneurons (Glausier and Lewis, 2011). Other GABA<sub>A</sub> receptor subunits are also altered in schizophrenia;  $\alpha 2$  mRNA levels are higher (Beneyto et al., 2011), while mRNA levels of  $\alpha 5$  (Duncan et al., 2010; Beneyto et al., 2011) and  $\delta$  (Hashimoto et al., 2008b; Maldonado-Avilés et al., 2009) are lower.

Thus, schizophrenia is thought to be characterized by alterations in both pre- and postsynaptic functioning of GABA neurons in the DLPFC. Markers of GABA synthesis and reuptake are lower, while levels of different GABA<sub>A</sub> receptor subunits are either increased or decreased. Taken together, these data point to altered GABA neurotransmission and subsequent impaired inhibition of postsynaptic pyramidal neurons.

### 1.3.3 Parvalbumin neurons are particularly affected in schizophrenia

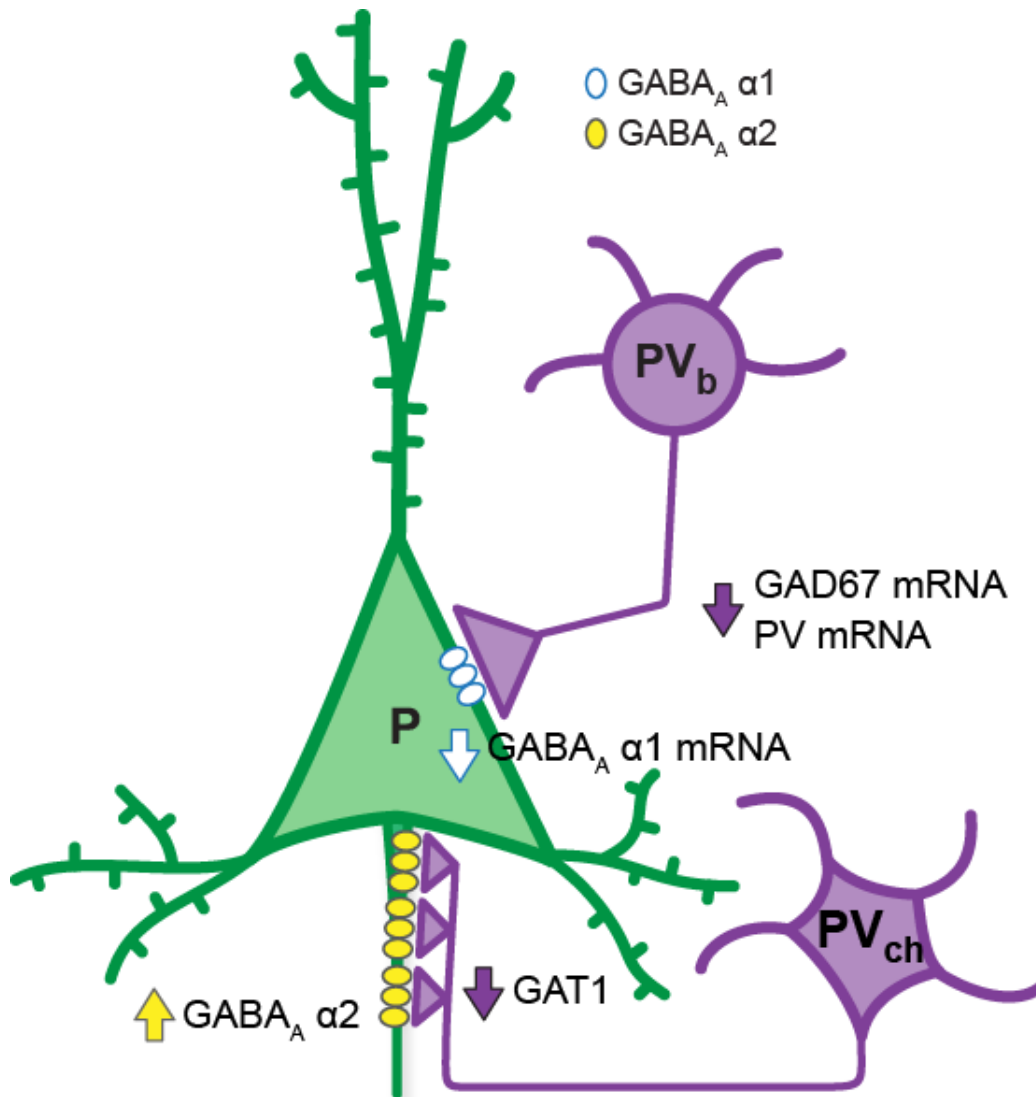
GABA neurons in the primate DLPFC can be divided into distinct subclasses based on their electrophysiological, molecular, and anatomical properties (reviewed in Gonzalez-Burgos and Lewis, 2008; Conde et al., 1994; Gabbott and Bacon, 1996; DeFelipe, 1997; Kawaguchi and Kubota, 1993; Krimer et al., 2005; Zaitsev et al., 2005). Electrophysiologically, interneurons can be divided into fast-spiking (**FS**) and non-fast spiking (**non-FS**) categories, based on membrane potential changes in response to current injection. FS cells contain the Ca<sup>2+</sup> binding protein PV, do not contain any neuropeptides, and target the soma, proximal dendrites, and axon initial segment of pyramidal cells (hereafter referred to as PV cells). Non-FS cells are more heterogeneous and are made up of several different subpopulations. One subpopulation expresses the Ca<sup>2+</sup> binding protein calbindin and the neuropeptide somatostatin (**SST**),

and targets the distal dendrites of pyramidal cells. Another subtype contains the  $\text{Ca}^{2+}$  binding protein calretinin (**CR**) and the neuropeptide vasoactive intestinal peptide (**VIP**), and mainly targets other interneurons. A third group does not express any  $\text{Ca}^{2+}$  binding proteins, contains the neuropeptide cholecystokinin (**CCK**) and the cannabinoid 1 receptor (**CB1**), and synapses onto the perisomatic region of pyramidal cells. Given the different electrical, molecular, and anatomical properties of the different subtypes of interneurons, the functional outcome of impaired GABA neurotransmission in schizophrenia depends on the subpopulation(s) of neurons affected.

PV neurons comprise ~25% of primate DLPFC GABA neurons (Conde et al., 1994) and can be divided into two main classes: 1) basket cells (**PV<sub>b</sub>**) that target the soma and proximal dendrites of pyramidal cells and 2) chandelier cells (**PV<sub>ch</sub>**) that target axon initial segments (**AIS**), forming distinct vertical arrays termed cartridges (Lewis and Lund, 1990). Axons of PV<sub>b</sub> neurons have a much larger spread than those of PV<sub>ch</sub> cells, and are much more numerous (Zaitsev et al., 2005). These two classes of PV cells also differ in the postsynaptic GABA<sub>A</sub> receptor subunits that predominate at their synapses: PV<sub>ch</sub> synapses preferentially express  $\alpha 2$ -containing GABA<sub>A</sub> receptors, while PV<sub>b</sub> synapses predominately contain  $\alpha 1$  receptors (Nusser et al., 1996).

PV cells are one subtype of GABA interneuron that contain lower GAD67 mRNA in schizophrenia. In the DLPFC of subjects with schizophrenia, dual label ISH has shown that approximately 50% of PV mRNA+ neurons lack detectable levels of GAD67 (Hashimoto et al., 2003). The expression of PV mRNA is significantly decreased (Hashimoto et al., 2003; Hashimoto et al., 2008b; Mellios et al., 2009), and laminar analyses have revealed that this reduction occurs in layers 3 and 4 (Hashimoto et al., 2003; Hashimoto et al., 2008b), layers in which GAD67 mRNA is also lower in schizophrenia. However, in contrast to GAD67 mRNA, although the expression of PV mRNA per neuron is decreased, neither the density of neurons with detectable PV mRNA levels (Hashimoto et al., 2003), nor the density of PV-IR neurons (Woo et al., 1997; Beasley et al., 2002) is changed. Furthermore, the within-pair PV mRNA expression per neuron is significantly correlated with change in density of GAD67 mRNA+ neurons (Hashimoto et al., 2003). These data suggest that PV neurons are not lost, but that GAD67 mRNA expression is reduced in PV-expressing neurons with lower,

although still detectable, levels of PV mRNA. Evidence suggests that both PV<sub>ch</sub> and PV<sub>b</sub> cells may contain less GAD67 mRNA. The density of PV<sub>ch</sub> cartridges that are immunoreactive for GAT1 is 40% lower in schizophrenia, although the density of other GAT1-IR structures is unaltered (Woo et al., 1998). Given that the changes in GAD67 and GAT1 seem to be present in the same neurons (Volk et al., 2001), this suggests that PV<sub>ch</sub> cells exhibit lower GAD67. Moreover, the number of GABA<sub>A</sub>  $\alpha$ 2-containing AIS is increased in the DLPFC, which has been interpreted as a compensatory response to lower GABA signaling in PV<sub>ch</sub> cells (Volk et al., 2002). Additionally, the density of PV-IR puncta, possibly the axon terminals of PV<sub>b</sub> cells, is 24% lower in the middle layers of the cortex (Lewis et al., 2001), suggesting that PV<sub>b</sub> cells may also exhibit a GAD67 deficit. Thus, alterations in a variety of markers of GABA neurotransmission are present in DLPFC of subjects with schizophrenia (**Figure 2**).



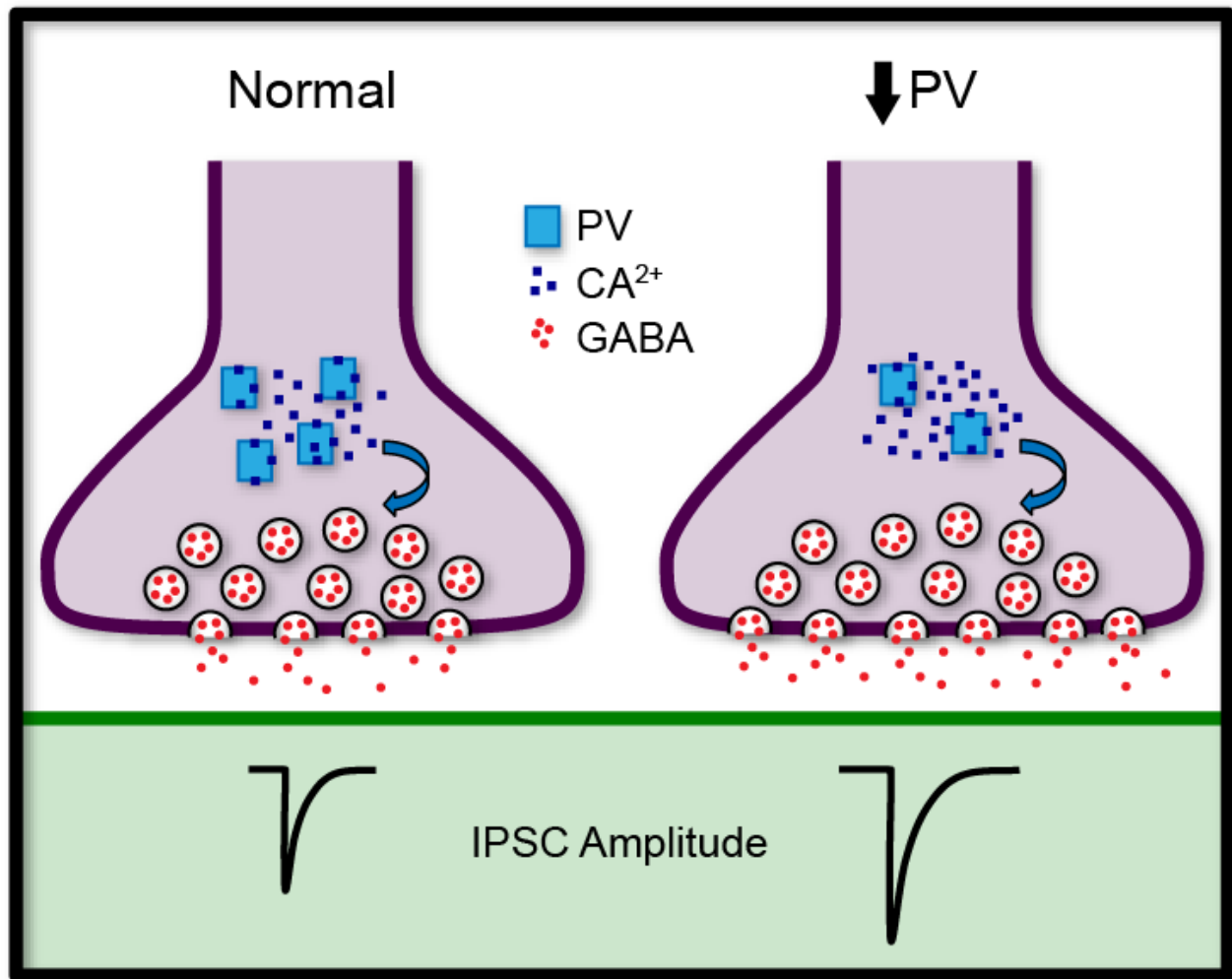
**Figure 2. Schematic of alterations in GABA neurotransmission markers in the DLPFC of subjects with schizophrenia.** Basket cells (PV<sub>b</sub>) contact the soma and proximal dendrites of pyramidal cells (P), which contain lower levels of GABA<sub>A</sub> α1 mRNA. Chandelier cells (PV<sub>ch</sub>) synapse exclusively onto the pyramidal cell AIS, which contains increased GABA<sub>A</sub> α2 and decreased GAT1 immunoreactivity. Both types of PV cells are thought to contain less GAD67 and PV.

Abnormalities in PV neurons alone may not account for the reductions in GAD67 mRNA. In addition to the observed reductions in layers 3-4 in the DLPFC, lower levels of GAD67 mRNA have also been observed in layers 1, 2, and 5, where relatively few PV-expressing GABA neurons are located and where PV mRNA expression is unaltered (Hashimoto et al., 2003). This suggests that other subsets of interneurons residing outside layers 3 and 4 may also exhibit reductions in GAD67. One candidate population is those neurons expressing CCK and CB1. Lower levels of CCK mRNA (Hashimoto et al., 2008a) and CB1 mRNA and protein (Eggan et al., 2008; Hashimoto et al., 2008a) have been found in the DLPFC of subjects with schizophrenia, and changes in GAD67, CCK, and CB1 mRNA are significantly correlated in the same subject pairs (Eggan et al., 2008). A third population of interneurons thought to be affected in the illness is those expressing SST. SST mRNA is reduced in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2008a; Morris et al., 2008; Hashimoto et al., 2008b), and the reductions are significantly correlated with the deficit observed in GAD67 mRNA in the same subject cohort. In contrast, the 50% of cortical interneurons that express CR (Conde et al., 1994) do not seem to be affected in schizophrenia, as there are no changes in CR mRNA, -IR neurons, or -IR axon terminals in schizophrenia subjects relative to comparison subjects (Hashimoto et al., 2003; Hashimoto et al., 2008b; Woo et al., 1997; Cotter et al., 2002).

#### **1.3.4 Lower PV and GAT1 in schizophrenia: compensation for lower GAD67 in PV interneurons?**

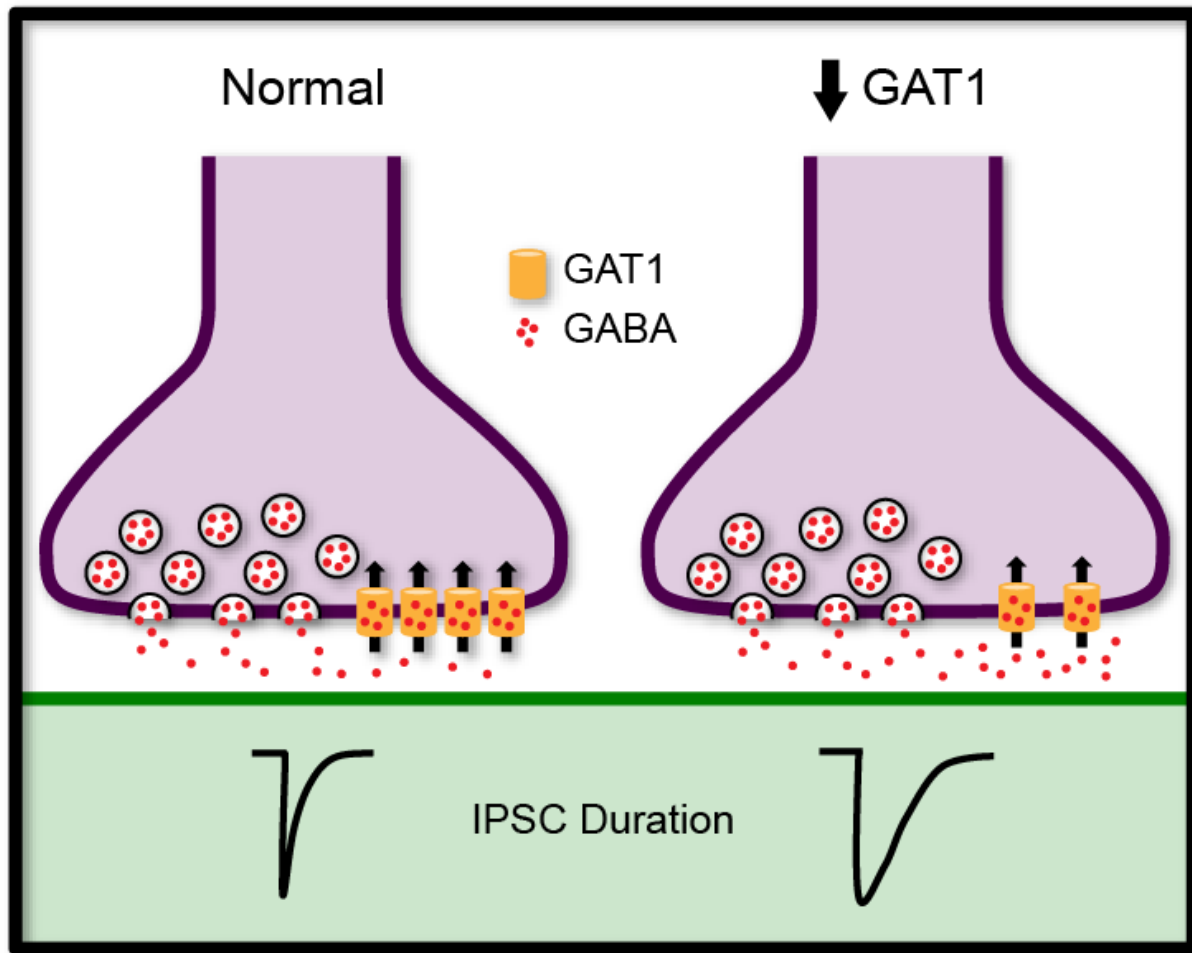
Reductions in PV in the DLPFC of subjects with schizophrenia may represent a compensatory response to a deficit in GAD67 (Lewis et al., 2005). Neurotransmitter is released from vesicles at chemical synapses following an influx of  $\text{Ca}^{2+}$  ions into the nerve terminal through voltage-gated  $\text{Ca}^{2+}$  channels. PV, a member of the E-F hand family of  $\text{Ca}^{2+}$  binding proteins, is a slow  $\text{Ca}^{2+}$  buffer that increases the rate at which residual  $\text{Ca}^{2+}$  transients are decayed in GABA nerve terminals after neurotransmitter release (Collin et al., 2005; Muller et al., 2007). Therefore, during repetitive firing, reduced PV levels should result in increased GABA release (**Figure 3**), and, in fact, PV-

deficient mice do exhibit enhanced facilitation of GABA release and increased IPSP amplitude from fast-spiking neurons (Collin et al., 2005; Vreugdenhil et al., 2003).



**Figure 3. Effect of lower PV on GABA release and postsynaptic functioning.** In the presence of normal PV levels (left),  $Ca^{2+}$  released following repetitive firing is sequestered, leading to lower levels available to stimulate GABA release, and producing a normal inhibitory postsynaptic current (**IPSC**) amplitude. In the presence of reduced PV levels (right), more  $Ca^{2+}$  is available to promote GABA release, resulting in increased GABA release and a larger IPSC amplitude.

Reductions in GAT1 in the DLPFC of subjects with schizophrenia are also thought to be a compensation for reduced GAD67 (Lewis et al., 2005). GAT1 is one of four high-affinity  $\text{Na}^+/\text{Cl}^-$  transporters that take up extracellular GABA, and is the most abundant GABA transporter in the cortex. It is expressed on the plasma membranes of primarily neurons but also some astrocytes (Conti et al., 1998; Minelli et al., 1995). The majority of GAT1 protein is localized to interneuron axon terminals that contact pyramidal cells, though it is also observed in terminals that contact other interneurons (Minelli et al., 1995; Conti et al., 1998). Consistent with a role for GAT1 in removing GABA from synapses,  $^3\text{[GABA]}$  uptake is virtually absent in the hippocampus of GAT1 knockout (**KO**) mice (Jensen et al., 2003). Additionally, tonic  $\text{GABA}_A$  receptor-mediated current is increased substantially in the hippocampus after treatment with a GAT1 inhibitor (Nusser and Mody, 2002), and in GAT1 KO mice (Jensen et al., 2003). When nearby synapses are activated synchronously, blockade of GAT1 results in longer evoked IPSCs (Overstreet and Westbrook, 2003) (**Figure 4**), which suggests that a reduction in GAT1 in schizophrenia would maximize the effect of the existing, presumably lower, GABA levels.



**Figure 4. Effect of lower GAT1 on GABA release and postsynaptic functioning.** In the presence of normal GAT1 levels (left), some of the GABA released into the synaptic cleft is removed via GAT1, producing a normal IPSC duration. In the presence of reduced GAT1 levels (right), GABA remains in the synaptic cleft for longer, prolonging the postsynaptic IPSC.

Importantly, given that schizophrenia is characterized by deficits in DLPFC function and cognitive control, the hypothesized compensations of PV and GAT1 are presumably insufficient to completely counteract lower GAD67 and restore GABA signaling to normal levels. Thus, GABA levels are still lower than in healthy individuals, and functional impairments remain.



### **1.3.5 Alterations in GABA neurons are specific to the disease process of schizophrenia**

An important consideration is whether the alterations in markers of GABA neurotransmission described above are specific to the disease process of schizophrenia, or may be a consequence of having, or being treated for, the illness (Lewis et al., 2005). First, due to the large proportion of patients receiving antipsychotics, as well as antidepressants, benzodiazepines, and valproic acid due to the presence of comorbid depression and anxiety (reviewed in Green et al., 2003), the effects of these medications on markers of GABA neurotransmission need to be evaluated. Indeed, studies have demonstrated that monkeys treated with clinically-relevant doses of antipsychotic medications do not have lower levels of GAD67 (Volk et al., 2000), PV (Hashimoto et al., 2003), and GAT1 (Volk et al., 2001) mRNAs. In addition, no effect of benzodiazepines or valproic acid was observed in any of these transcripts (Hashimoto et al., 2008b). Second, although schizophrenia patients experience prevalent comorbid substance abuse (Green et al., 2003), alcohol use does not seem to affect GAD67, PV, and GAT1 mRNA levels (Volk et al., 2000; Volk et al., 2001; Hashimoto et al., 2003; Hashimoto et al., 2008b), although the effect of nicotine and cannabis use have not yet been examined. However, one limitation of the analyses that have been performed to date is that small sample sizes that have resulted from parsing the cohorts into smaller groups to examine these potentially confounding variables. Thus, a large study is needed to confirm these findings.

## **1.4 GOALS AND RELEVANCE OF THIS DISSERTATION**

Although many studies have replicated lower mean GAD67 mRNA in the DLPFC of subjects with schizophrenia, several aspects of this deficit are currently unknown, and a number of important questions remain unanswered. First, given the substantial heterogeneity in GAD67 mRNA levels across subjects with schizophrenia, just how common is a GAD67 mRNA deficit? Second, what sources may contribute to the

heterogeneity in GAD67 mRNA levels? For example, such variability may result from potential confounds such as medication use, substance abuse, or illness chronicity. Alternatively, since lower GAD67 is thought to underlie the cognitive deficits of the illness (Lewis et al., 2005), which are known to be the best predictors of functional outcome (Green et al., 2000), there may be a relationship between GAD67 mRNA levels and indices of illness severity and functional outcome. Third, since mRNA levels are not necessarily predictive protein levels, does the mRNA deficit extend to the protein level? Fourth, if there is a protein deficit, does it occur specifically in PV neurons like the mRNA deficit? Fifth, what are the levels of GAD67 protein at the principal site of GABA production and function, the axon terminal? Finally, what are the consequences of a reduction of GAD67 in PV cells? Accordingly, this dissertation measured GAD67 mRNA, tissue-level protein, and axon terminal protein in PV cells, and examined the consequences of lower GAD67 protein in PV neurons. These data provide an extensive characterization of the GAD67 deficit in the DLPFC of subjects with schizophrenia, and provide novel evidence of a functional impairment in PV neurons that is thought to underlie cognitive impairments.

Since the measurement of protein levels in schizophrenia was associated with significant challenges, **Chapter 2** describes these difficulties and the strategies used to overcome them. The following two experimental sections (**Chapters 3 and 4**) address the questions raised above. **Chapter 5** concludes with a discussion and interpretation of the data.

## **2.0 STRATEGIES TO OVERCOME THE METHODOLOGICAL CHALLENGES OF ASSESSING PROTEIN LEVELS IN POSTMORTEM HUMAN TISSUE**

### **2.1 ABSTRACT**

In this dissertation, using gray matter from dorsolateral prefrontal cortex (DLPFC) area 9 in schizophrenia subjects, we measured 1) total GAD67 protein using Western blotting and 2) GAD67 protein levels in PV axon terminals using immunofluorescence. However, the assessment of protein levels in postmortem human tissue is challenging for a number of reasons. This chapter outlines these challenges and discusses the strategies used to overcome them. Since protein measures are confounded by postmortem interval (PMI), prior to assessing total GAD67 protein levels in schizophrenia we measured the effect of PMI on GAD67 protein in order to select a cohort of subjects with minimal GAD67 degradation. Additionally, due to substantial Western blotting variability, we utilized a study design that minimized and controlled for such variability, selected a stable normalizer, and determined an appropriate protein load that would ensure accurate quantification. In order to accurately assess the low GAD67 axon terminal protein levels predicted in schizophrenia, we characterized the sensitivity of our confocal system, developed an approach to apply stereology, and adapted a recently-developed threshold/morphological segmentation and data analysis approach to postmortem human tissue. Additionally, we developed a novel approach to eliminate the autofluorescence of lipofuscin, an intracellular protein that confounds fluorescent intensity measures. The data resulting from these approaches are presented in **Chapter 3**.

## 2.2 INTRODUCTION

Expression of mRNA is not necessarily predictive of protein levels (Tian et al., 2004), and it is the protein product of a gene that ultimately carries out its function. Thus, the examination of protein levels in schizophrenia provides an important complement to mRNA studies. In this dissertation two measures of GAD67 protein in DLPFC area 9 gray matter in postmortem human tissue were made: 1) total protein by Western blot and 2) axon terminal protein within PV neurons by immunofluorescence. Prior to these assessments, several methodological hurdles first needed to be overcome. Here, these challenges are outlined and the strategies we utilized to surmount them are presented. The development and implication of these strategies formed a significant portion of this dissertation, and thus they are highlighted here. The resulting data produced using these methodologies are presented in **Chapter 3**.

## 2.3 ASSESSMENT OF TOTAL GAD67 PROTEIN LEVELS USING WESTERN BLOTTING

### 2.3.1 Challenges

Measurement of total protein levels in postmortem human tissue using Western blotting is associated with two major challenges. First, protein measures are subject to several confounds. Most problematic is postmortem interval (**PMI; 2.3.2**), the time between death and processing of the tissue, which can have variable effects on different proteins (reviewed in Lewis, 2002). For example, PMI effects can differ substantially between members of the same family of proteins (Hayes et al., 1991), and between brain regions (Irving et al., 1997). Therefore, we first assessed the effect of PMI on GAD67 protein in DLPFC tissue prior to examining GAD67 protein in schizophrenia, in order to select a cohort of subjects with PMIs associated with minimal GAD67 degradation. A second major challenge we faced, to our surprise, was that Western blotting of postmortem

human tissue was associated with substantial variability, particularly across gels. Reliable quantification of human postmortem Western blotting data requires a strategy to minimize the effects of this variation. Accordingly, we employed a study design that was able to restrict, and control for, such variation **(2.3.3)**. Third, the use of a normalizer protein in Western blotting is important to control for differences in the amount of protein loaded between wells; however, this normalizer must not only be stable across multiple experiments and subjects, but also unaltered in schizophrenia. Therefore, we performed a comparison of two commonly used normalizers **(2.3.4)**. Fourth, the amount of protein loaded per well needs to be within the range of linearity and proportionality of the antibodies utilized in order to ensure accurate quantification. Accordingly, we characterized both antibodies utilized in order to determine an appropriate protein load **(2.3.5)**.

### **2.3.2 Effect of postmortem interval on GAD67 protein levels**

To assess the effects of PMI on GAD67 protein levels, we used tissue samples from 6 male, white healthy subjects, aged 53-55 years. Subjects were divided into 2 triads, each containing a subject with a short, medium, and long PMI (Triad 1 PMIs: 8.0, 15.5, and 23.2 hrs; Triad 2 PMIs: 6.4, 15.4, and 28.0 hrs). Members of a triad were processed together throughout all steps. Tissue collection, tissue preparation, and Western blotting was performed as described in **Appendix A**. Mean (standard deviation; **SD**) tubulin-corrected (see **2.3.4** for selection of normalizer) GAD67 optical densities (**ODs**) for the short, medium, and long PMIs were 0.49 (0.12), 0.43 (0.10), and 0.40 (0.10), respectively. GAD67 protein levels were reasonably well-preserved across PMI, with 88 and 82% of the level present in the short PMI group (7.2 hrs) still present at 16 and 26 hrs, respectively.

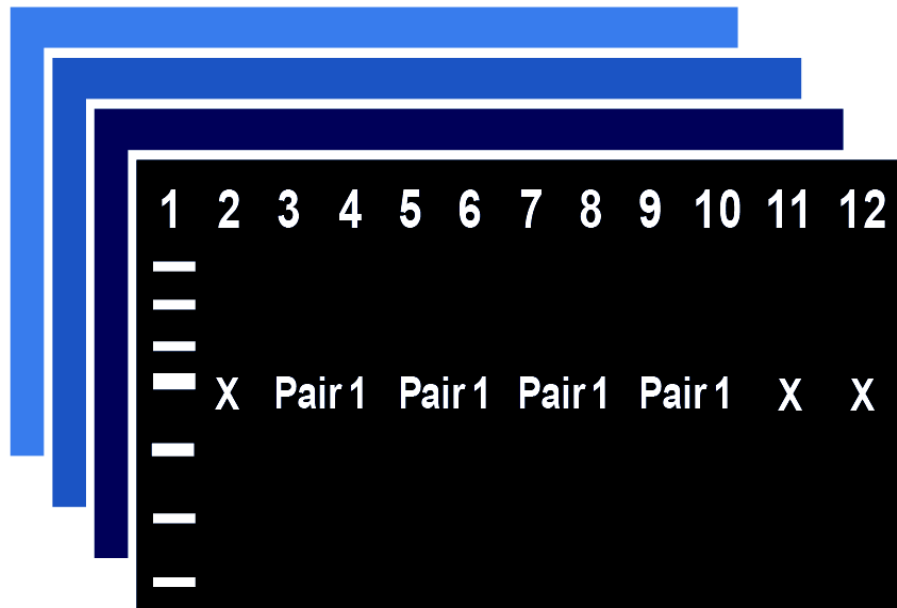
In order to verify and extend the human findings, we performed a similar analysis in DLPFC tissue from an adult male macaque monkey in which *in vivo* levels (i.e., PMI of 0 hr) could be obtained. Monkey DLPFC tissue was collected as described previously (Hayes et al., 1991). Briefly, the animal was sedated with ketamine hydrochloride, intubated, anesthetized with 1% halothane in 28% oxygen, and placed in

a stereotaxic apparatus. The skull was opened and biopsy blocks were removed from the PFC. A single block containing the DLPFC was frozen immediately in isopentane (0 hr PMI), and 3 others were stored in room temperature artificial cerebrospinal fluid for 6, 12, or 24 hrs and then frozen (6, 12, and 24 hr PMI). Tissue sections were collected from the blocks on a cryostat, protein was extracted, and samples prepared for Western blotting as described in **Appendix A**.

Mean (SD) tubulin-corrected GAD67 ODs for the 0, 6, 12, and 24 hr PMIs were 0.39 (0.05), 0.30 (0.04), 0.27 (0.04), and 0.30 (0.05), respectively. GAD67 protein levels in monkey DLPFC were relatively well-preserved across all PMIs with 78, 69, and 76% of the baseline GAD67 level retained at the 6, 12, and 24 hr time points, respectively. However, the greater loss of GAD67 protein across PMI in the monkey (in which comparison was made to a true 0 hr PMI) relative to the human indicates that the real effect of PMI on GAD67 protein in human tissue may be slightly larger than what was observed. Consequently, we used a more stringent cut off than 24 hours, and selected pairs of schizophrenia and comparison subjects with PMIs < 20 hrs for subsequent GAD67 protein quantification.

### **2.3.3 Minimizing sources of variability with experimental design**

We found that Western blotting of human postmortem tissue is associated with substantial variability, especially across gels. In order to make quantitative measures, an appropriate gel loading design is needed to minimize, and account for, this variability. Since we observed the greatest variability in signal at the edges of a gel (lanes 1, 2, 11, and 12 of a 12-well gel), we utilized only the middle 8 lanes for quantification. Additionally, we made a total of 16 replicate measures for each subject pair using 4 gels, on which the subject pair was loaded 4 times (**Figure 5**). This approach maximized the number of replicates of a single pair per gel, thereby minimizing within-gel variability. In addition, replicate gels were processed within the same experimental run to further reduce variability. Since all data for a given subject pair were collected in the same run, and all measures per subject were averaged together, the within-pair comparison controlled for the remaining variability.



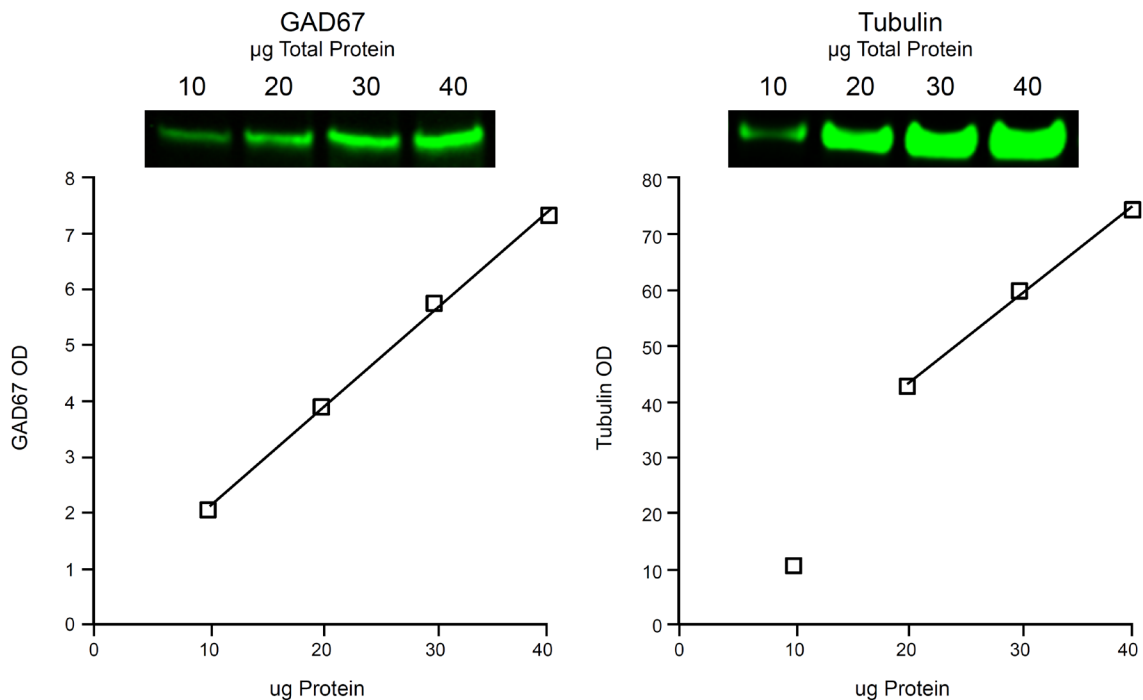
**Figure 5. Gel loading design.** A single pair of subjects was loaded per gel. Four identical gels were loaded per pair and run simultaneously, for a total of 16 replicates per subject. The molecular weight marker was loaded in the far left lane. Xs mark lanes that were not used for quantification.

#### 2.3.4 Selection of normalizer protein

To maximize our ability to account for potential differences in the amount of protein loaded per well, we measured the levels of two commonly used normalizers,  $\beta$ -actin and  $\beta$ -tubulin. Mean values for both proteins did not differ between the schizophrenia and comparison subjects ( $n=15$  pairs), but the coefficient of variation (**CV**) of the within-pair difference was 3 times greater for  $\beta$ -actin (0.104) than for  $\beta$ -tubulin (0.036). Therefore, we corrected each GAD67 OD measure by the  $\beta$ -tubulin OD measure from the same lane.

### 2.3.5 Selection of protein load

To determine an appropriate protein load, we assessed the linearity and proportionality of the GAD67 and  $\beta$ -tubulin antibodies as a function of total protein loads of 10, 20, 30, and 40  $\mu$ g (**Figure 6**). Assessing the linearity of the antibodies is possible due to the use of linear detectors in the LI-COR system. GAD67 and  $\beta$ -tubulin OD increased in a highly linear ( $r=0.99$ ,  $p=0.001$ ;  $r=0.99$ ,  $p=0.026$ , respectively) and proportional fashion as protein load increased from 10-40 and 20-40  $\mu$ g, respectively. Accordingly, we loaded each lane with 25  $\mu$ g of total protein, which is within the range of linearity and proportionality of both antibodies.



**Figure 6. GAD67 (1:800) and tubulin (1:20,000) OD increase in a linear and proportional fashion as protein load increases between 20 and 40  $\mu$ g.** The gel images and plots show an increase in GAD67 (left) and tubulin (right) OD with increasing protein load. OD and  $\mu$ g protein are significantly correlated from 10-40  $\mu$ g ( $r=0.99$ ,  $p=0.001$ ) and 20-40  $\mu$ g ( $r=0.99$ ,  $p=0.026$ ) for GAD67 and tubulin, respectively.



Abbreviations: OD, optical density. Note that despite their appearance, none of the bands of the tubulin blot are saturated.

## **2.4 ASSESSMENT OF GAD67 PROTEIN LEVELS IN PV AXON TERMINALS USING IMMUNOFLOUORESCENCE, CONFOCAL MICROSCOPY, AND ITERATIVE THRESHOLD/MORPHOLOGICAL SEGMENTATION**

### **2.4.1 Challenges**

The majority of research on markers of GABA neurotransmission in schizophrenia has focused on whole cell studies that examine mRNA expression and protein levels (Hashimoto et al., 2005; Guidotti et al., 2000), without looking specifically at the major site of function of these proteins, the axon terminal. In order to comprehensively evaluate effectors of GABA neurotransmission in schizophrenia, the axon terminal must be examined. Multilabel fluorescence confocal microscopy provides a means to assess relative levels of fluorescence per axon terminal, and by looking at several proteins simultaneously, it is possible to examine cell-type specificity of GAD67 alterations, which thus far has only been assessed in schizophrenia in one mRNA study (Hashimoto et al., 2003). However, quantifying protein levels in axon terminals in postmortem human brain tissue is challenging for four main reasons. First, postmortem tissue, especially that from a disease state such as schizophrenia, is associated with widely varying fluorescence levels. Thus, it is important to utilize a microscopy system with adequate sensitivity to detect very low levels of fluorescence, and we therefore characterized the sensitivity of our confocal system **(2.4.2.1)**. Second, because our confocal microscope was not equipped with stereological software, we generated a method to perform unbiased stereological imaging site selection **(2.4.2.2)**. Third, in order to assess cell-type specificity, we adapted a recently-developed fluorescence intensity/morphological segmentation **(2.4.2.3)** and data analysis **(2.4.3.4)** approach for use in human tissue in order to quantify GAD67 protein levels specifically in PV

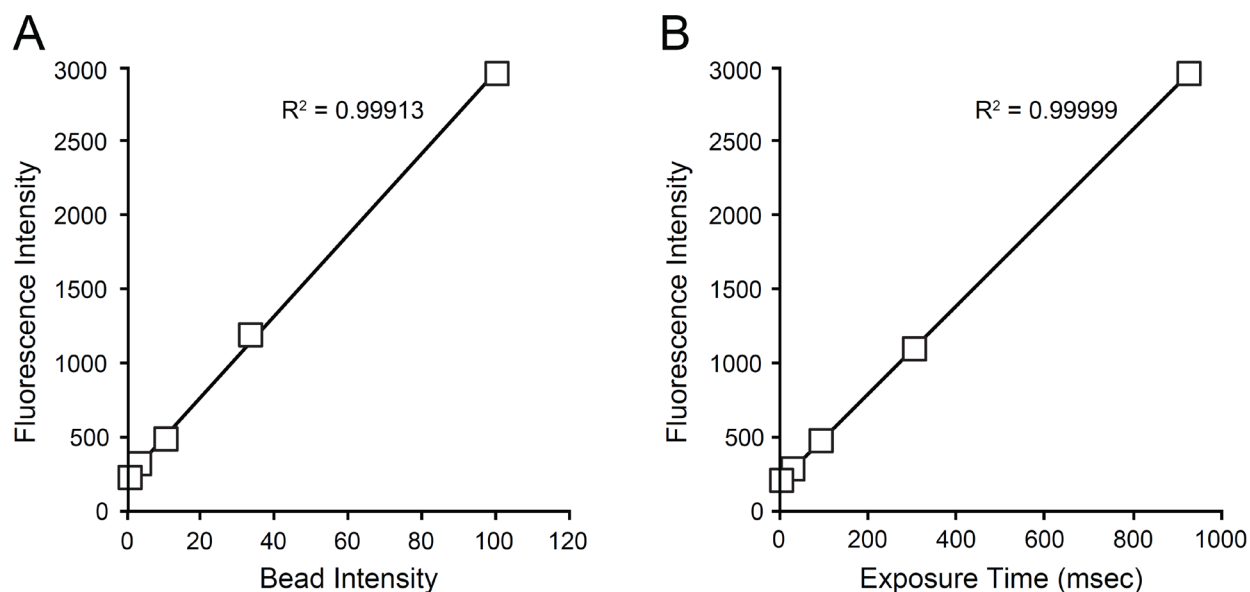
terminals. A fourth challenge associated with the measurement of axon terminal protein levels in postmortem human tissue is noise induced by the broad autofluorescence of lipofuscin, an intracellular lysosomal protein that accumulates with age in human cortex (Brody, 1960). Due to this autofluorescence, it has been extremely difficult, if not impossible, to differentiate “real” signal of the fluorophores of interest from lipofuscin signal. To overcome this problem, we developed an approach to eliminate the lipofuscin signal **(2.4.3)**.

## **2.4.2 Quantification of axon terminal protein levels**

### **2.4.2.1 Sensitivity of the microscopy system**

A major challenge in the assessment of axon terminal protein levels in postmortem tissue is the need to quantify a large range of fluorescence levels. For example, in monkey DLPFC tissue, GAD67 protein levels exhibit a wide range of intensities (Fish et al., 2011). Furthermore, in schizophrenia tissue, where GAD67 mRNA levels in a population of PV neurons fell below detectability using dual ISH, protein levels are predicted to be especially low (Hashimoto et al., 2003). Thus, a microscopy system with a high degree of sensitivity is needed. After processing human DLPFC tissue sections for fluorescence immunocytochemical labeling of inhibitory axon terminal markers (GAD65, GAD67, and PV) **(Appendix A)**, images were collected using an Olympus spinning disk confocal system (Center Valley, PA) using a 60X 1.42 N.A. oil immersion objective and a Hamamatsu C9100 Electron Multiplier Charge Coupled Device camera (Bridgewater, NJ). Using this system, when imaging fluorescence microspheres (beads) of varying relative intensities, we were able to successfully identify fluorescence intensity as low as 1.1X background. Additionally, with this system fluorescence intensity is highly linear as a function of both bead intensity and exposure time **(Figure 7)**, which allows for the accurate quantification of relative fluorescence levels over the dynamic range of the system. Accordingly, puncta with average fluorescence intensities barely over background and those with extremely high levels of fluorescence are identified equally well. Importantly, given that we hypothesize that schizophrenia subjects exhibit lower levels of GAD67 protein relative to comparison subjects, these

data suggest that this system is able to identify puncta with extremely low levels of protein.



**Figure 7. Fluorescence intensity is linear as a function of fluorescent microsphere intensity (A) and exposure time (B).** A: The high degree of linearity of fluorescence intensity across microspheres (beads) of varying intensity at the same exposure demonstrates the ability of our system to function within a wide range of axon terminal intensities. B: The high degree of linearity of fluorescence intensity across exposure times allows each image to be captured at optimal exposures for that site. Corrections for differences in exposure times can then be made during analysis.

#### 2.4.2.2 Sampling strategy

Due to the lack of stereological capabilities on the confocal system, we developed an alternative approach to sample the tissue in an unbiased, stereological fashion. First, the pia and white matter borders were traced on a separate microscope equipped with Stereoinvestigator software (MicroBrightField, Williston, VT). A region of interest

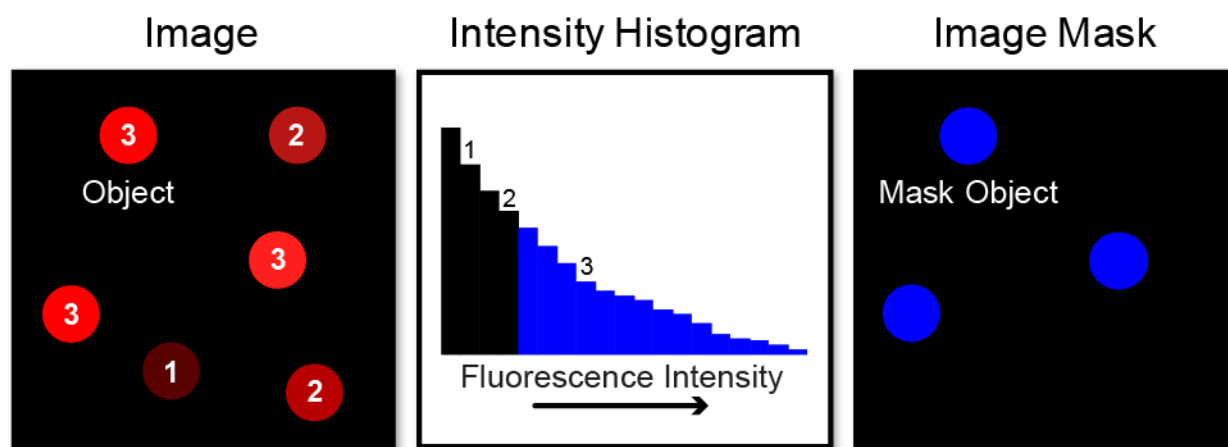
containing layers 3-4 (defined as 30-60% of the pia to WM distance) was identified in the largest continuous region with parallel pial and white matter borders. Next, the contours were printed onto a transparency, aligned with the tissue section using a 4X objective, and affixed to the top of each slide. Using the transparency, the microscope was driven to the center of the region of interest and a program written in Microsoft Excel was used to designate all possible sampling sites based on a randomly oriented grid. Due to limitations in the Stereoinvestigator software, the region of interest was constrained to a rectangular shape. As a result, a randomly oriented grid produced widely varying numbers of sampling sites within the region of interest across tissue sections. Therefore the maximum number of sites generated by the grid, up to 12, was sampled. Preliminary analyses indicated this number was sufficient to adequately sample the regions of interest, and after data collection we were able to show that all sections were adequately sampled using running means. The microscope was driven to the center of each optical dissector, and consecutive 512 x 512 pixel images (0.23  $\mu$ m apart) were collected starting from a position that was 75% the thickness of the tissue section from the coverslip and ending at the coverslip (see **Appendix A** for additional details). Images were assembled into 3D sets.

#### **2.4.2.3 Post-image processing and fluorescence intensity/morphological segmentation**

Post-processing and data segmentation were performed using a recently-developed methodology that allows for the quantification of fluorescently-labeled puncta, the colocalization of different labels in the same puncta, and the quantification of fluorescence intensity in these same structures (Fish et al., 2008). Since image stacks were taken using optimal capture settings (best image quality/no saturated pixels), the mode of the histogram was subtracted from each pixel to control for differences in background fluorescence intensity across subjects, sections, and image stacks (Sweet et al., 2010). This method is a more unbiased, consistent way of dealing with differences in background intensity between subjects, sections, and image stacks than selecting regions within each image stack. However, the subtracted value used might not fully represent the background level. The fluorescence intensity was corrected

based on exposure time (see **Figure 7B**). In addition, at the time, all available objectives were color-corrected only from 450-620 nm. Two of the utilized channels were outside this range, resulting in an axial shift that was corrected for using TetraSpeck 0.1  $\mu\text{m}$  microspheres (fluorescent blue/green/orange/dark red; Invitrogen). Importantly, the step size was chosen based on the need for correction.

Threshold-based segmentation can be used to identify structures of interest in an image, in which a threshold value is applied to the fluorescence intensity histogram of the data set. This threshold can either be determined visually (assigning voxels above and below the threshold a separate color, e.g., black and blue) or by a thresholding algorithm. This creates a virtual layer of data in which all voxels are reassigned a binary value according to whether or not they exceed the threshold. The resulting virtual layer is referred to as the image mask, composed of individual mask objects (**Figure 8**). For a 3D data set, the number of such object masks provides a count of the underlying objects within the image stack. For each mask object, data such as mean and total fluorescence intensity in each wavelength detected, volume, and center of volume can be generated (Sweet et al., 2010).



**Figure 8. Illustration of threshold segmentation.** A single threshold is applied to the image, so that all voxels are reassigned a binary value according to whether they fall below (black bars; 1 and 2) or above (blue bars; 3) the threshold. Objects within an image (left) are selected according to a threshold value of the fluorescence intensity

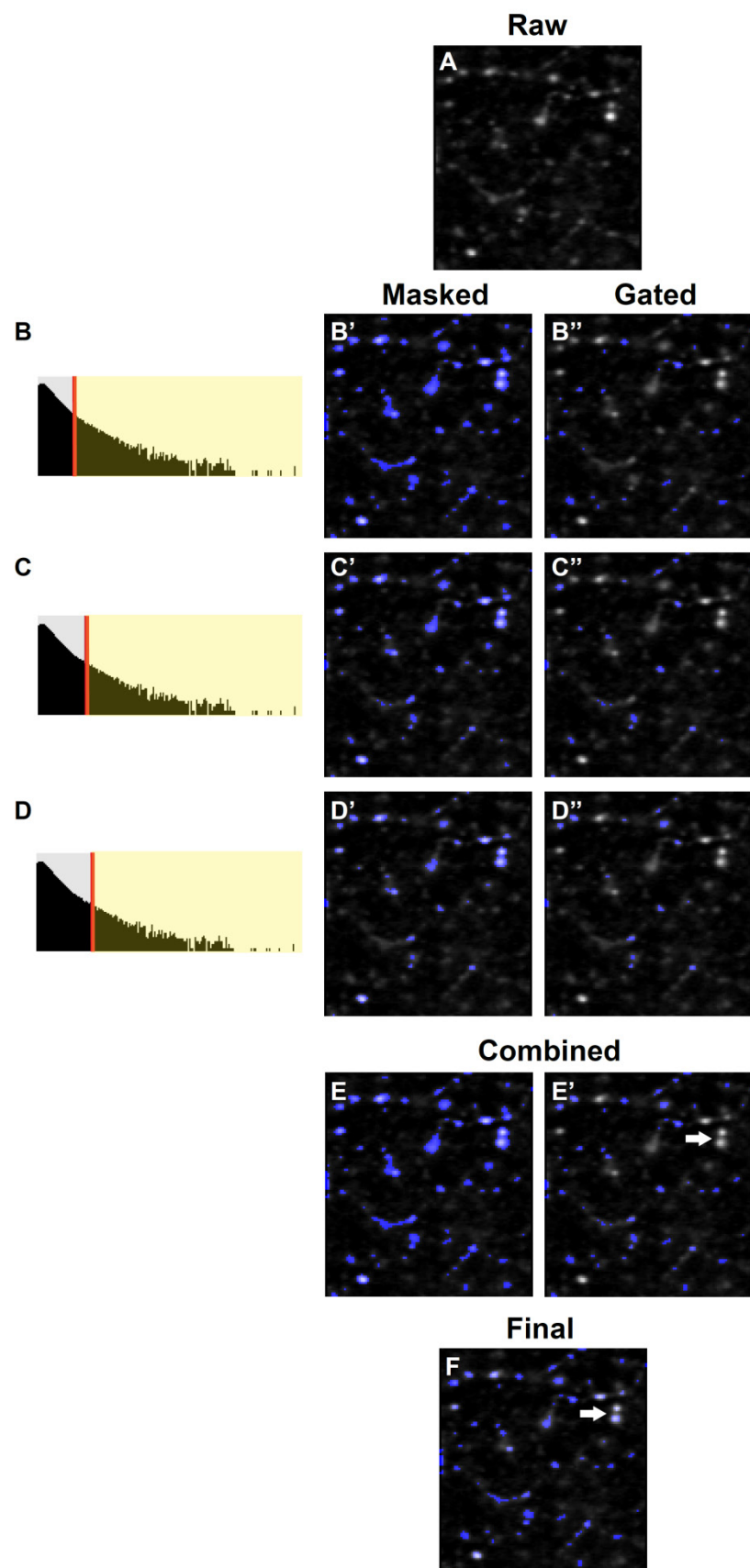
histogram (middle). All pixels above the threshold value are pseudocolored blue and selected for further analyses (right).

However, because terminal protein levels can vary naturally, with disease, or due to postmortem effects in human samples, a single threshold approach is unsuitable to mask all of the terminals of interest, and to precisely cover just those terminals that are masked, in postmortem schizophrenia tissue. As such, this single-threshold approach is not able to adequately measure fluorescence in both low- and high-expressing structures, and thus an alternative approach is needed.

To identify axon terminals, we utilized a recently-developed approach that combines information from multiple iterations of the single threshold approach, in which the low threshold value progressively migrates toward the high value (Fish et al., 2008). This methodology is able to mask puncta with high fluorescence levels equally as well as puncta exhibiting low fluorescence levels, and the final mask shapes are highly representative of the original data. When compared with other available semi-automated and automated software packages, this iterative approach was found to identify twice as many puncta and produced a much more uniform set of mask objects with final 3D ovoid shapes representative of true terminal shape (Fish et al., 2008).

Because the density of GAD65-IR axon terminals is not altered in schizophrenia (Benes et al., 2000), GAD65 immunoreactivity was used to identify IR puncta, putative axon terminals. The final processed data set was threshold segmented, masked, and objects in the mask were selected by size using a range of 0.06 to 0.7  $\mu\text{m}^3$  (**Figure 9**). This size range was empirically determined and corresponded to previously published methods (Fish et al., 2008; Fish et al., 2011). We performed 60 iterations of this intensity segmentation/mask/morphological segmentation protocol using different low threshold settings that progressively migrated towards the high value, which remained fixed. The number of iterations was determined empirically. As observed in **Figure 9**, as the iterations progress, lower intensity mask objects are no longer collected in favor of higher intensity objects. However, after the second iteration, the resulting size-gated

mask objects were combined with those from the first cycle into a single mask such that completely overlapping objects were represented by one mask object. This new mask containing the combined mask objects was then combined with those selected in the third iteration. This process of combining mask objects into a single mask continued throughout all iterations. Thus, early iterations were used to identify lower-intensity objects, while later iterations identified high-intensity objects and, due to the combining of objects from all iterations, the final mask therefore contained a relatively uniform set of object masks that approached the upper size limit of the selection criteria.





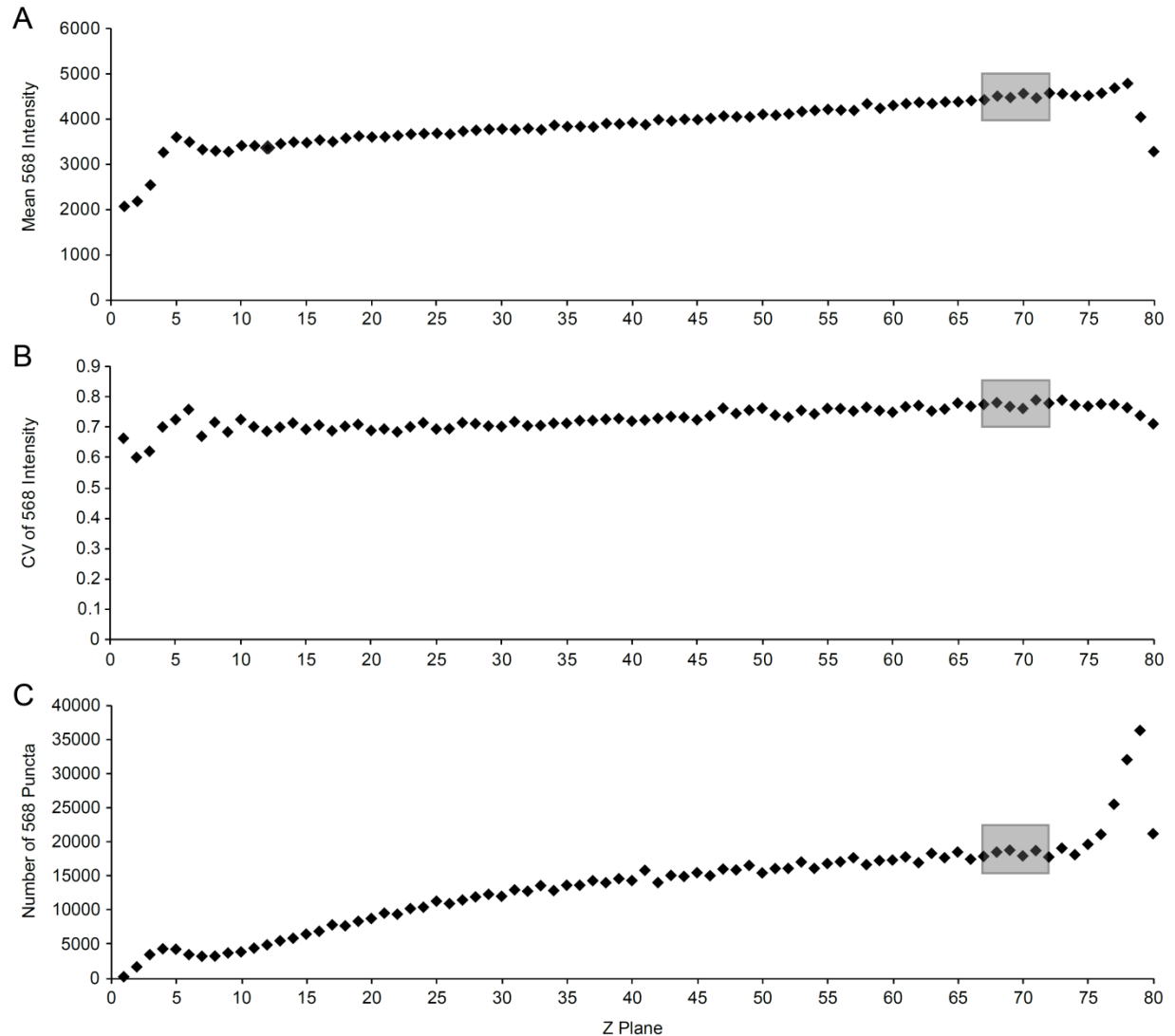
**Figure 9. Illustration of the iterative threshold/morphological segmentation approach.** Images are generated from a single z-plane. Raw data is shown in greyscale, selected data at each step is shown in blue. In the first iteration, a raw image (A) is threshold segmented according to the histogram in B, and intensities within the yellow highlighted region are selected, generating the blue mask objects in B'. Only selected objects are retained after size gating (B''). The threshold intensity value progressively migrates toward the maximum intensity and the threshold/morphological segmentation process is repeated in the second (C, C', C'') and third (D, D', D'') iterations. As the iterations advance, the mask objects generated are combined (E and E'). After just 3 iterations, the resultant mask objects (E'') are highly representative of the original data. In the final data set (F), generated using 60 iterations, all terminals are successfully masked, including those not identified using 3 iterations (designated by the arrow in E' and F).

#### **2.4.2.4 Data Analysis**

After the iterative masking approach, analyses were performed to select only the data of interest. In order to eliminate potential confounds due to edge effects and antibody penetration, only selected regions of data in the x, y, and z axes were utilized in quantification. Additionally, only puncta IR for all 3 markers (GAD65, PV, and GAD67) were selected for final analyses. Finally, any masks containing fluorescence resulting from lipofuscin were eliminated, as described in **2.4.5**.

Mask objects falling within a 10-pixel border at the extreme x and y edges (pixels 1-10 and 502-512 in both x and y) of each image were discarded to prevent edge effects. Given differences in antibody penetration across the z axis, only a portion of the z axis was selected for analysis. The upper and lower ~1  $\mu\text{m}$  of z planes, selected based on the maximum observed terminal size, were excluded due to irregularity in the surface of the tissue and compression at the slide/coverslip. Fluorescence intensity, and thus puncta number, is greatest at the coverslip due to minimal blur (since there is the least amount of tissue to image through) and maximal antibody penetration. Therefore, the z plane located closest to the coverslip (after exclusion of ~1  $\mu\text{m}$  as described

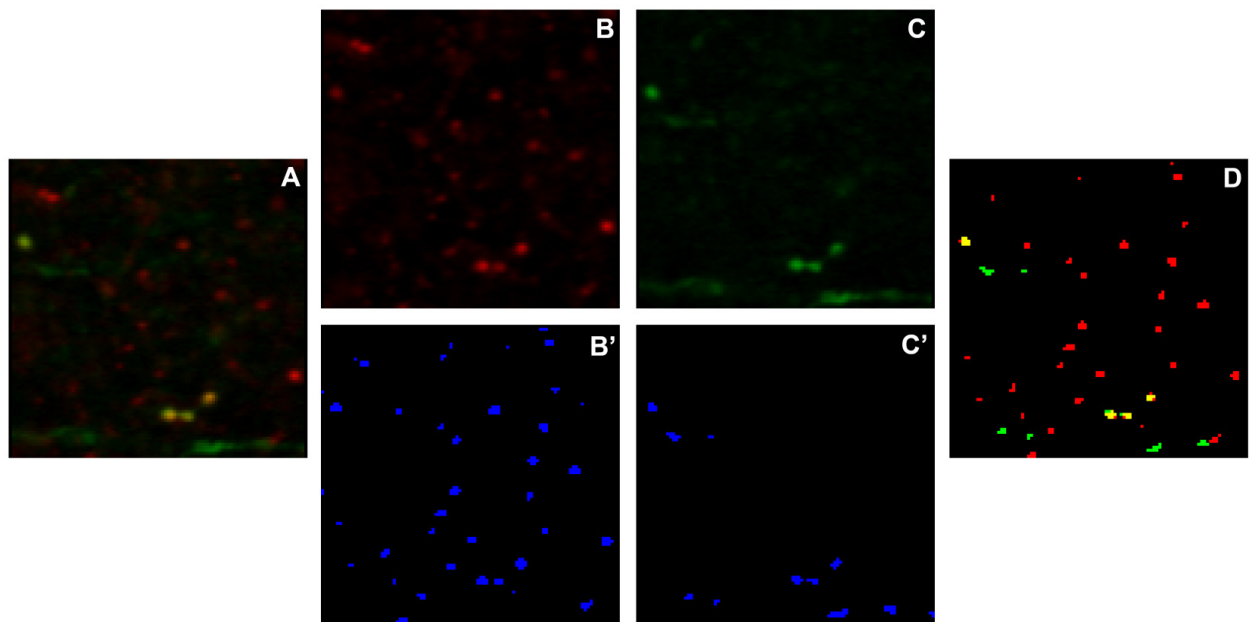
above) was selected as the starting point of the quantified region. Regression analyses were performed as planes were progressively added from the starting point to determine the largest number of z planes for which the slopes of the mean GAD65 intensity, CV of mean GAD65 intensity, and number of GAD65 puncta regression lines were not statistically significantly different from 0 (all  $F \leq 2.884$  all  $p \geq 0.165$ ) (**Figure 10**). The selected region comprised 8% of the total thickness of the tissue.



**Figure 10. Determination of region of z axis quantified.** Plots of mean intensity (A), CV of intensity (B), and number of puncta (C) across the z axis in the GAD65 (568) channel. Regression analyses were performed to determine the region of z planes of which the slopes of each line were not statistically significant from 0. The shaded boxes depict the selected region utilized in quantification.

Mask operations between channels were used to determine GAD65-IR puncta that were also -IR for PV (colocalized), and GAD67 intensity was quantified within GAD65+/PV+ terminals (**Figure 11**). In order to be classified as GAD65+/PV+, 1) a

minimum overlap between masks objects needed to be  $\geq 4$  voxels (the smallest allowable puncta size) and 2) the nonoverlapping voxels needed to comprise  $< 25\%$  of the PV mask object volume. This second, more stringent, criterion was used to eliminate cases in which the signals were not truly colocalized despite a GAD65 and PV signal overlap  $\geq 4$  voxels. No statistical difference ( $t_4=0.998$ ,  $p=0.375$ ) between the pairwise percent difference in GAD67 protein using criterion 1 compared to both criteria was observed. However, it is easy to imagine cases in which the results would significantly differ (e.g. in which one terminal type is always next to another type, thus producing bleed over). Accordingly, we utilized the more stringent criteria (1 and 2) in the final dataset.



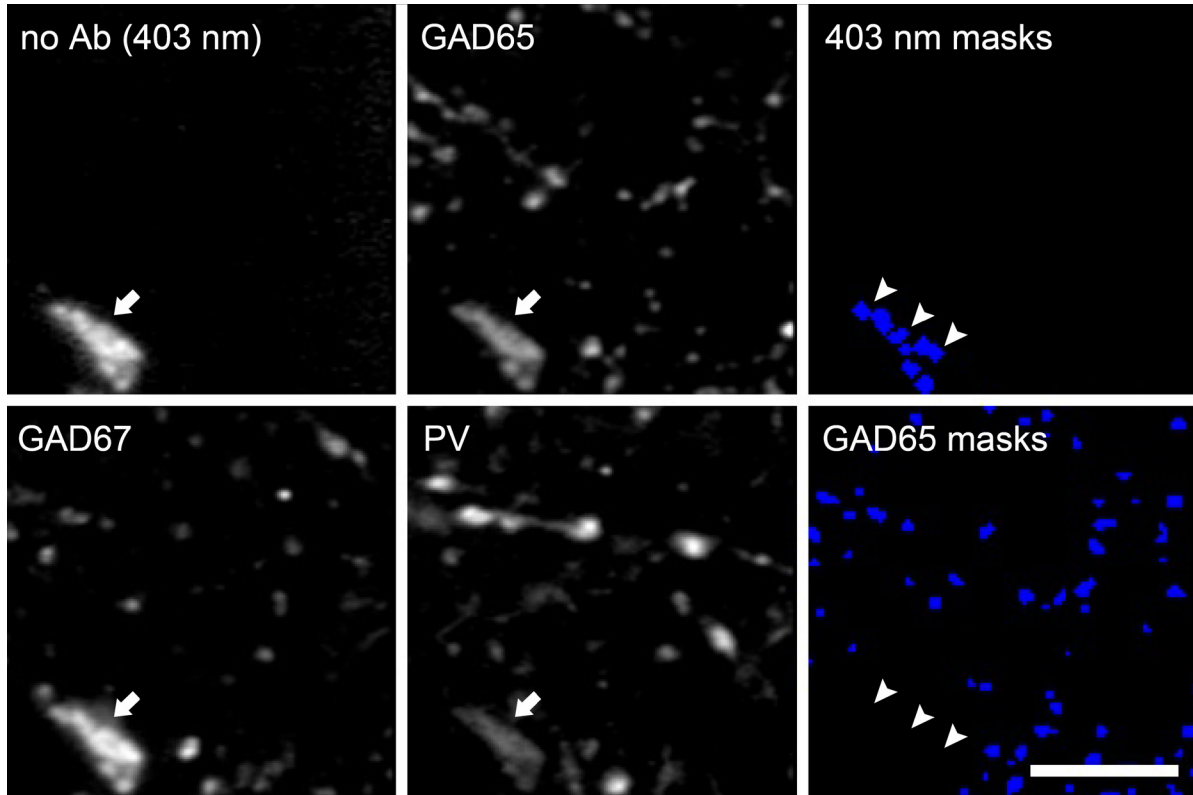
**Figure 11. Illustration of GAD65 and PV colocalization.** Images are generated from a single z-plane. The raw image of both GAD65-IR (red) and PV-IR (green) axon terminals (A) can be separated into single channels (B and C, respectively). Mask objects from GAD65-IR (B') and PV-IR (C') structures were generated as described in **Figure 9**. The overlay of GAD65-IR (red) and PV-IR (green) mask objects (D) demonstrates the colocalization (yellow) that was used to determine structures that contained both GAD65 and PV.

### 2.4.3 Elimination of confounding lipofuscin autofluorescence

A potential confound of fluorescent intensity measures in human cortex is the broad and intense autofluorescence lipofuscin. Lipofuscin is an intracellular lysosomal protein that accumulates with age in many brain regions, including the cortex (Porta, 2002; Brody, 1960), and autofluoresces across the usable spectrum of the microscope (~350-700 nm) (Gray and Woulfe, 2005). At 5 years of age, lipofuscin is present in less than 5% of human cortical neurons, but is found in virtually all neurons by the third decade of life (Benavides et al., 2002). The number of lipofuscin granules per cell also increases with age, and the rate of lipofuscin accumulation is negatively correlated with longevity (reviewed in Brunk and Terman, 2002), earning it the nickname of “aging pigment.” Lipofuscin is composed primarily of protein, but also contains about 30% lipid, <10% carbohydrate, as well as traces amounts of metals such as iron, copper and zinc (Double et al., 2008). One prominent theory is that lipofuscin accumulates as the result of oxidative stress. Reactive oxygen species may result in crosslinking of proteins and lipids, producing cellular components that are indigestible by lysosomal enzymes (Chio et al., 1969; Chio and Tappel, 1969; Brunk and Terman, 2002). Schiff bases, 1,4-dihydropyridines, and 2-hydroxy-1,2-dihydropyrrol-3-ones are thought to be responsible for the autofluorescent properties of lipofuscin.

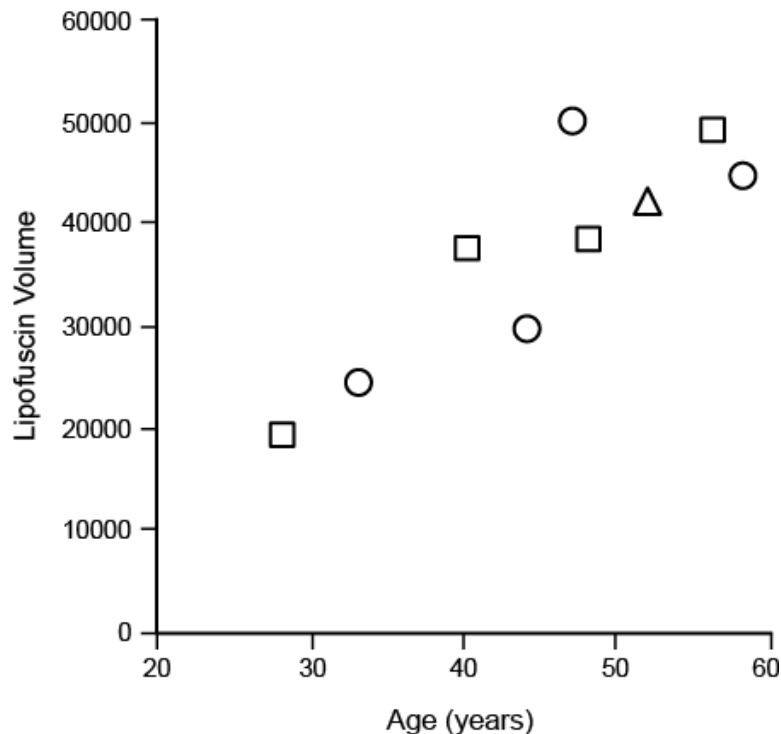
As shown in **Figure 12**, we detected lipofuscin signal in all 3 channels of interest. Our masking approach can erroneously identify bright spots of lipofuscin signal as axon terminals, and thus lipofuscin represents a significant confound of axon terminal fluorescence levels. Accordingly, we developed a novel approach to exclude lipofuscin autofluorescence from our analyses, and thus eliminate the potential confounds it presents. Lipofuscin was imaged in the 403 nm channel (which does not overlap with channels used to image GAD65, GAD67, or PV) and then masked for subsequent quantification and exclusion from the GAD67 data (**Figure 12**). Due to high signal intensity, the mask objects used to quantify and subsequently exclude the 403 nm signal were dilated by a 1-pixel diameter in 3D to ensure that the entirety of the signal was masked. Any GAD67+/GAD65+/PV+ puncta that overlapped with 403 nm masks were excluded. In addition, a 403 nm maximum intensity cut off of 200 was applied to

the data set to eliminate any lipofuscin that was not masked by our automated approach.



**Figure 12. Illustration of lipofuscin autofluorescence.** The arrow in the single z-plane images shows lipofuscin autofluorescence in the 403 (top left), GAD65 (top middle), GAD67 (bottom left), and PV (bottom middle) channels. To exclude lipofuscin signal, any GAD65 mask objects that overlapped with 403 nm mask objects (arrowheads; top right) were removed from final analyses (bottom right). Scale bar=10 $\mu$ m.

Consistent with previous studies (Benavides et al., 2002; Brody, 1960), subject age was significantly correlated with total lipofuscin volume ( $r=0.87$ ,  $p=0.002$ ) (**Figure 13**). Additionally, the total volume of masked lipofuscin did not significantly differ between comparison and schizophrenia subjects ( $t_4=-1.0$ ,  $p=0.366$ ), and lipofuscin volume per subject was not significantly correlated with GAD67 intensity in PV puncta ( $r=0.17$ ,  $p=0.641$ ). Importantly, these data demonstrate that our approach successfully excluded lipofuscin from our analyses and did not confound measures of GAD67 intensity.



**Figure 13. Lipofuscin volume correlates with subject age.** The plot shows the age of comparison (square), schizophrenia (circle), and schizoaffective (triangle) subjects against the total lipofuscin volume per subject. Subject age is significantly correlated with total lipofuscin volume ( $r=0.87$ ,  $p=0.002$ ). One outlier subject was excluded.

## 2.5 CONCLUSIONS

The approaches presented here represent innovative solutions to the challenges associated with measurement of protein in postmortem human tissue. The quantitative Western blotting strategy provides a robust quantification of total GAD67 protein due to the use of only subjects with PMIs in which GAD67 protein is well-preserved, a paired study design with multiple replicates, a stable normalizer, and a protein load within the range of linearity and proportionality of the antibodies utilized. Immunofluorescence and confocal microscopy, coupled with a novel methodology, provides a robust assessment of GAD67 protein levels in a specific population of interneurons due to the use of a sensitive microscopy system, stereological sampling, an iterative threshold/morphological segmentation and data analysis approach that allows for the accurate quantification of a wide range of fluorescent intensities, and the elimination of the confounding autofluorescence of lipofuscin. Importantly, this marks the first time this approach has been utilized in postmortem human tissue. The total GAD67 protein and PV axon terminal GAD67 protein levels in schizophrenia resulting from these two approaches are presented in **Chapter 3**.



### **3.0 CORTICAL DEFICITS OF GLUTAMIC ACID DECARBOXYLASE 67 EXPRESSION IN SCHIZOPHRENIA: CLINICAL, PROTEIN, AND CELL TYPE- SPECIFIC FEATURES**

Curley AA, Arion D, Volk DW, Asafu-Adjei JK, Sampson AR, Fish KN, Lewis DA (2011) Cortical Deficits of Glutamic Acid Decarboxylase 67 Expression in Schizophrenia: Clinical, Protein, and Cell Type-Specific Features. *Am J Psychiatry*, in press. Reprinted with permission from the American Journal of Psychiatry (copyright 2011). American Psychiatric Association.

#### **3.1 ABSTRACT**

Cognitive deficits in schizophrenia are associated with altered activity of the dorsolateral prefrontal cortex (DLPFC), which has been attributed to lower expression of the 67 kDa isoform of glutamic acid decarboxylase (GAD67), the major GABA-synthesizing enzyme. However, little is known about the relationship of prefrontal GAD67 mRNA levels and illness severity, translation of the transcript into protein, or protein levels in axon terminals, the key site of GABA production and function. Quantitative PCR was used to measure GAD67 mRNA levels in the DLPFC from 42 subjects with schizophrenia and 42 matched comparison subjects. Western blotting was used to quantify tissue levels of GAD67 protein in a subset of subjects where potential confounds of protein measures were controlled. Multi-label, confocal immunofluorescence was used to quantify GAD67 protein levels in the axon terminals of parvalbumin (PV)-containing GABA neurons, known to have low levels of GAD67 mRNA in schizophrenia. GAD67 mRNA levels were significantly 15% lower in schizophrenia subjects, but transcript levels were not associated with predictors or

measures of disease severity or chronicity. In schizophrenia subjects, GAD67 protein levels were significantly 10% lower in total gray matter and 49% lower in PV axon terminals. These findings, that lower GAD67 mRNA expression is common in schizophrenia, is not a consequence of having the illness, and leads to less translation of the protein, especially in the axon terminals of PV-containing neurons, support the hypothesis that lower GABA synthesis in PV neurons contributes to DLPFC dysfunction and impaired cognition in schizophrenia.

### **3.2 INTRODUCTION**

Impairments in cognition are a core feature of schizophrenia and the best predictor of functional outcome (Green, 1996). The cognitive deficits span multiple domains (Keefe and Fenton, 2007), suggesting that they reflect an overarching alteration in cognitive control, the ability to adjust thoughts or behaviors in order to achieve goals (Lesh et al., 2011). Cognitive control depends on the coordinated activity of a number of brain regions, including the DLPFC, which plays an integral role in the maintenance and selection of rules that govern behavioral responses (Miller and Cohen, 2001). Subjects with schizophrenia exhibit altered activation of the DLPFC (Minzenberg et al., 2009), and DLPFC dysfunction is correlated with cognitive impairment (Weinberger et al., 1986).

Frontal lobe  $\gamma$  band (30-80 Hz) oscillations, which appear to underlie higher-order cognitive processes (Tallon-Baudry et al., 1998), are positively associated with cognitive control processes in comparison subjects, but subjects with schizophrenia fail to exhibit the normal task-related increase in  $\gamma$  band power (Cho et al., 2006; Minzenberg et al., 2010). Oscillations in the  $\gamma$  frequency are known to depend on the firing of GABA neurons (Gonzalez-Burgos and Lewis, 2008), and thus alterations in DLPFC GABA neurotransmission may contribute to impaired cognition in schizophrenia (Lewis et al., 2005).

GABA signaling is regulated by levels of its synthesizing enzyme, GAD. The 67 kDa isoform, GAD67, is responsible for the majority of GABA synthesis in the cortex

(Mason et al., 2001), whereas the 65 kDa isoform, GAD65, contributes to GABA synthesis principally during conditions of high synaptic demand (Patel et al., 2006). Consistent with this interpretation, reducing GAD67 expression in mice results in lower GAD activity and GABA content (Asada et al., 1997), but reducing GAD65 expression does not significantly alter cortical GABA levels (Asada et al., 1996). Lower GAD67 mRNA levels in the DLPFC have been consistently reported in schizophrenia (Gonzalez-Burgos et al., 2010). However, the relationship between GAD67 mRNA expression and the clinical features of the illness has not been examined in a large sample. Furthermore, since GAD67 expression is activity-dependent (Benson et al., 1994), lower expression might just index the less stimulating social, occupational, and intellectual environment associated with illness chronicity. Accordingly, we quantified DLPFC GAD67 mRNA levels in 42 subjects with schizophrenia and matched comparison subjects, and examined their relationship to predictors of illness severity, measures of functional outcome, and duration of illness.

Although lower GAD67 mRNA levels in the DLPFC are consistently found in schizophrenia, how this finding relates to levels of the cognate protein remains unclear despite a single prior study reporting lower mean levels of both GAD67 mRNA and protein in schizophrenia (Guidotti et al., 2000). This question is of particular importance given that mRNA and protein levels are not necessarily correlated, since a number of factors regulate transcription and translation (Nelson and Keller, 2007). For example, pharmacological manipulation of GABA levels is associated with changes in GAD67 protein but not mRNA (Rimvall et al., 1993). Furthermore, measures of protein in postmortem human tissue are subject to confounds that do not influence transcript measures (Beneyto et al., 2009). Thus, we employed a quantitative Western blotting strategy that minimized sources of variability to test the hypothesis that cortical GAD67 protein levels are lower in schizophrenia.

Understanding the functional significance of altered GAD67 protein levels requires knowledge of the affected cell class. In schizophrenia, GAD67 mRNA levels are markedly lower only in about 25-35% of interneurons (Akbarian et al., 1995a; Volk et al., 2000), including the PV-containing GABA neurons (Hashimoto et al., 2003) that are crucial to the generation of  $\gamma$  oscillations (Cardin et al., 2009). Thus, if lower GAD67

mRNA and protein levels in PV neurons underlie the impaired  $\gamma$  band oscillations and cognitive control deficits in schizophrenia (Gonzalez-Burgos and Lewis, 2008), then lower GAD67 protein should be particularly apparent in PV cells at the principal site of GABA production and function, the axon terminal. Accordingly, we used quantitative fluorescence imaging (Fish et al., 2008) in a proof-of-concept test of the idea that GAD67 protein levels are much lower in PV axon terminals than in total cortical gray matter.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Human Subjects

Brain specimens (n=84) were obtained during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA) after consent was obtained from the next of kin. Consensus DSM IV diagnoses for each subject were made by a committee of experienced clinicians on the basis of medical records and the results of structured interviews conducted with family members of the deceased (Hashimoto et al., 2008b). To reduce biological variance between groups and control for experimental variation, each schizophrenia subject was matched with one comparison subject for sex, and as closely as possible for age and PMI, and tissue samples from members of a pair were always processed together. Comparison subjects had no known history of neurological or psychiatric illness. Tissue was collected from Brodmann's area 9 and samples were prepared as described in **Appendix A**. All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

For quantification of GAD67 mRNA levels in schizophrenia, tissue from all 42 subject pairs was utilized. For every subject, the RNA integrity number (**RIN**) was  $>7$ , which is associated with excellent RNA quality (Imbeaud et al., 2005). Mean age, PMI, brain pH, RIN, and tissue storage time were not different for the two subject groups (all  $t_{82} \leq 2.0$ ,  $p \geq 0.051$ ) (**Table 1**; for individual subject characteristics, see **Appendix B**). For

measures of total GAD67 protein, only the subject pairs (n=19) in which both members had PMIs less than 20 hrs (see **3.4.2** for rationale) were used (**Table 1**). Of these 19 pairs, 5 pairs were selected for the PV axon terminal study (see **3.4.4** for rationale) (**Table 1**).

**Table 1. Summary of demographic characteristics for each study<sup>a</sup>**

	qPCR		Western blotting		Immunofluorescence	
	C	S	C	S	C	S
N	42	42	19	19	5	5
Sex	31M/11F	31M/11F	14M/5F	14M/5F	5M	5M
Race	34W/8B	29W/13B	14W/5B	14W/5B	5W	5W
Age (years)	48±13	47±13	44±13	43±11	45±12	47±9
PMI (hours)	17.8±5.9	18.1±8.7	12.5±4	11.4±5.4	14.3±3.6	12.3±4.7
Brain pH	6.8±0.2	6.6±0.4	6.8±0.2	6.5±0.3	6.8±0.2	6.5±0.4
RIN	8.3±0.6	8.2±0.7	8.5±0.6	8.2±0.6	8.6±0.4	8.1±0.6
ST (months)	97±43	97±46	95±45	99±44	116±16	110±23

<sup>a</sup>Values are group means±standard deviation. For each study, none of the between-group comparisons were statistically significantly different, except for pH in the Western blotting study ( $t_{36}=3.0$ ,  $p=0.005$ ). Abbreviations: C, comparison; S, schizophrenia; PMI, postmortem interval; RIN, RNA integrity number; ST, freezer storage time.

### 3.3.2 GAD67 mRNA

GAD67 mRNA levels were assessed by qPCR using the comparative threshold cycle measurement method (Hashimoto et al., 2008b). Four replicate measures were performed for each subject with a standard threshold for each transcript consistently applied across all subjects. Based on their stable level of expression between schizophrenia and comparison subjects (Hashimoto et al., 2008b), 3 reference genes

[ $\beta$ -actin, cyclophilin A, and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**)] were used to normalize GAD67 mRNA levels.

### **3.3.3 Total GAD67 protein levels**

Western blotting was performed as described in **Appendix A**. Bands were measured in arbitrary OD units using the Odyssey® Infrared Imaging System software (LI-COR Biosciences, Lincoln, NE) by a single investigator (AAC). Red (680 nm) and green (800 nm) channels were quantified separately. OD was measured by drawing a rectangle around each band. The size of the rectangle was adjusted to include the entire band without including signal from neighboring bands. Each band was measured 3 times with a new rectangle. Background OD was measured according to LI-COR specifications.

In an effort to reduce, and account for, the within- and between-subject measurement variance, replicate measures for each subject pair were made both within and across gels. Samples from members of a pair were loaded in adjacent lanes of the same gel. Each pair of samples was loaded 4 times on a gel, and 4 replicate gels were run per pair and processed simultaneously (i.e. 16 replicate measures). Of the resulting 608 data points, 28 were dropped before analysis due to obvious gel artifacts, with no more than 2 of the 16 measures per subject excluded.

### **3.3.4 GAD67 protein levels in PV puncta**

Fluorescence immunohistochemistry was used to label 2 tissue sections per subject with antibodies against GAD65, GAD67, and PV (see **Appendix A**). Using a previously published method (Sweet et al., 2009), a region within area 9 where the tissue section was cut perpendicular to the pial surface was identified. Sampling sites were then placed in this region in a systematic random fashion within layers 3-4, defined as 30-60% of the pia-to-white matter distance (Eggan et al., 2008). The GAD67 mRNA deficit in PV neurons in schizophrenia is most pronounced in these layers (Hashimoto et al., 2003). Image stacks (n=180) were collected from the 5 subject pairs on a confocal microscope as described in **Appendix A**.

Because the density of GAD65-IR axon terminals is not altered in schizophrenia (Benes et al., 2000), GAD65 immunoreactivity was used to identify IR puncta (defined as labeled structures 0.06–0.7  $\mu\text{m}^3$  in size) that represent putative axon terminals. Post-processing and data segmentation were performed using a methodology that allows for the quantification of fluorescently-labeled puncta, the colocalization of different labels in the same puncta, and the quantification of fluorescence intensity in these same structures (Fish et al., 2008). In this method, a binary channel (mask) is made of the segmented pixels and used to assess fluorescence intensity. Each mask is composed of multiple mask objects (IR puncta). Mask operations between channels were used to determine GAD65-IR puncta that were also labeled for PV and GAD67.

### 3.3.5 Statistical analyses

Two analysis of covariance (**ANCOVA**) models were performed to test the effect of diagnosis on GAD67 mRNA level. The first model included mRNA level as the dependent variable, diagnostic group as the main effect, subject pair as a blocking factor, and storage time, brain pH, and RIN as covariates. Subject pairing may be considered an attempt to balance diagnostic groups for sex, age, and PMI, and to account for the parallel processing of tissue samples from a pair, and thus not a true statistical paired design. Therefore, we also used a second model without subject pair as a blocking factor that included sex, age, PMI, storage time, brain pH, and RIN as covariates.

A similar approach was used for total protein measures of GAD67, with appropriate adjustments made for covariates. However, to account for the across-gel measurement variability inherent in Western blotting, samples from a single subject pair comprised all lanes of a given gel (see **3.3.3**) and thus only a paired ANCOVA model was used. The use of a paired model is supported by the similar results obtained with both paired and unpaired ANCOVA analyses of GAD67 mRNA levels (see **3.4.1**).

Paired t tests were used to assess whether the GAD67 protein levels in PV puncta differed between comparison and schizophrenia subjects.

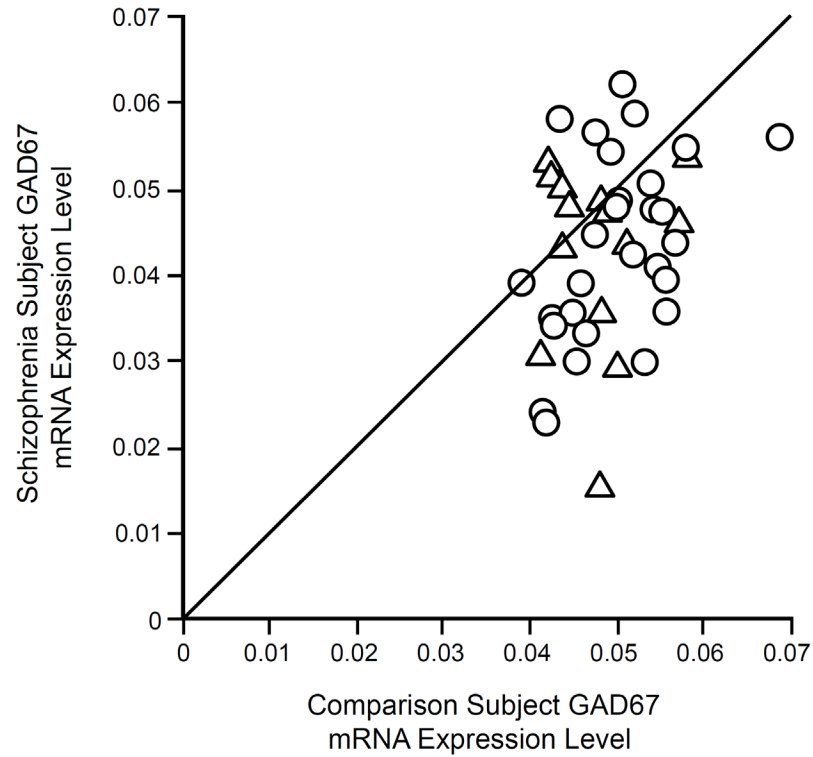
The reported p values for comparisons of GAD67 levels are one-tailed because mRNA levels have been previously shown to be lower in schizophrenia (Gonzalez-Burgos et al., 2010). All other p values are two-tailed.

### 3.4 RESULTS

#### 3.4.1 GAD67 mRNA expression in schizophrenia

Mean GAD67 mRNA levels were significantly 14.5% lower in the 42 subjects with schizophrenia relative to matched comparison subjects (paired:  $F_{1,38}=7.3$ ,  $p=0.005$ ; unpaired:  $F_{1,76}=6.5$ ,  $p=0.006$ ) (**Figure 14**). None of the covariates were significant in either of the models (all  $F \leq 1.5$ ,  $p \geq 0.226$ ) except for RIN (only in the paired model;  $F_{1,38}=6.4$ ,  $p=0.016$ ). In 23 of these pairs, lower GAD67 mRNA levels had previously been reported by ISH (using a riboprobe designed against a different portion of the mRNA than the primer set employed here) (Hashimoto et al., 2005). The pair-wise differences in GAD67 mRNA expression as measured by ISH and by qPCR in the present study were strongly positively correlated ( $r=0.68$ ,  $p=0.000$ ). The new set of 19 subject pairs also showed a significantly 12.3% lower level of GAD67 mRNA in schizophrenia (paired:  $F_{1,15}=4.1$ ,  $p=0.031$ ; unpaired:  $F_{1,30}=6.0$ ,  $p=0.010$ ).

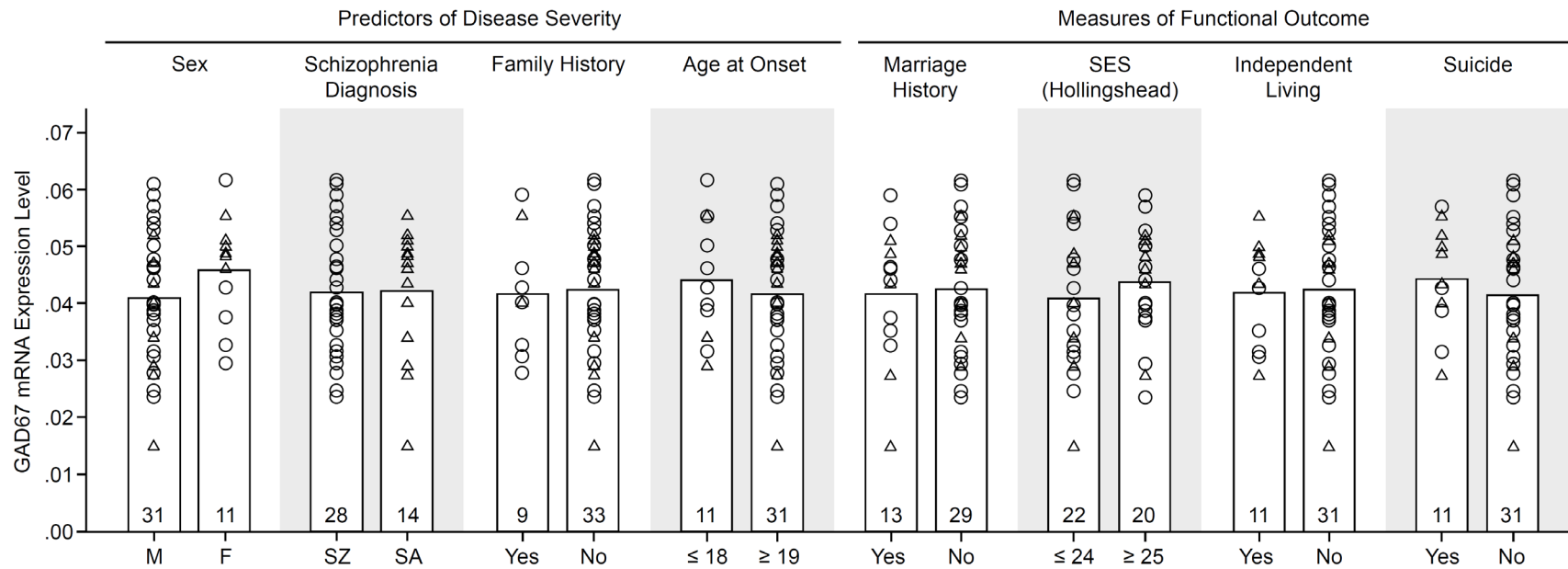
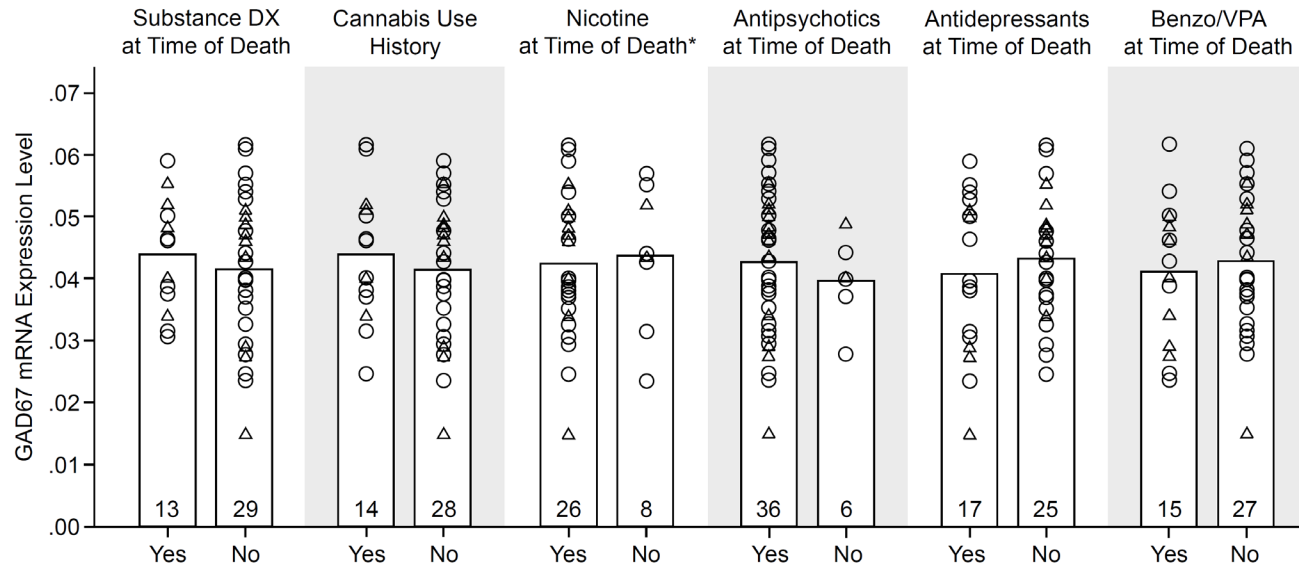




**Figure 14. GAD67 mRNA levels in schizophrenia and comparison subjects.** The scatter plot indicates GAD67 mRNA levels for each matched pair of healthy comparison subject and subject with schizophrenia (circles) or schizoffective disorder (triangles). Pairs below the diagonal unity line have lower GAD67 mRNA in the schizophrenia or schizoffective subject relative to the comparison subject. Levels of GAD67 mRNA were significantly ( $F_{1,38}=7.3$ ,  $p=0.005$ ) 14.5% lower in the schizophrenia subjects.

GAD67 mRNA levels in the schizophrenia subjects did not differ as a function of diagnosis of substance abuse or dependence current at the time of death (**ATOD**); use of antidepressants, benzodiazepines or sodium valproate, antipsychotics, or nicotine ATOD; or history of cannabis use (all  $p \geq 0.486$ ) (**Figure 15**). Additionally, regression models were built to look for significant interaction terms between the variables (with the exception of nicotine ATOD, because data were not available for all subject pairs) and a sequence of models with increasing interaction complexity was fit. A model with main effects and all two-way interactions revealed no significant effects (all  $p \geq 0.073$ ).

The within-pair percent differences in GAD67 mRNA in schizophrenia relative to comparison subjects were heterogeneous (range:-67% to +34%) and thus we examined several factors that might contribute to the variability. Among schizophrenia subjects, GAD67 mRNA levels did not differ (all  $p \geq 0.214$ ; **Figure 15**) as a function of factors that predict a more severe course of illness [male sex, a diagnosis of schizophrenia rather than schizoaffective disorder, first degree relative with schizophrenia, early age of onset (<19 years of age)], or measures of illness severity [suicide, no history of marriage, low socioeconomic status (**SES**) as measured by the Hollingshead Two Factor Index of Social Position, absence of independent living ATOD] (DSM-IV-TR, 2000). Additionally, GAD67 mRNA levels were not significantly correlated with age of onset ( $r=0.09$ ,  $p=0.561$ ), age ATOD ( $r=-0.25$ ,  $p=0.104$ ), or age-corrected length of illness ( $r=0.31$ ,  $p=0.134$ ).



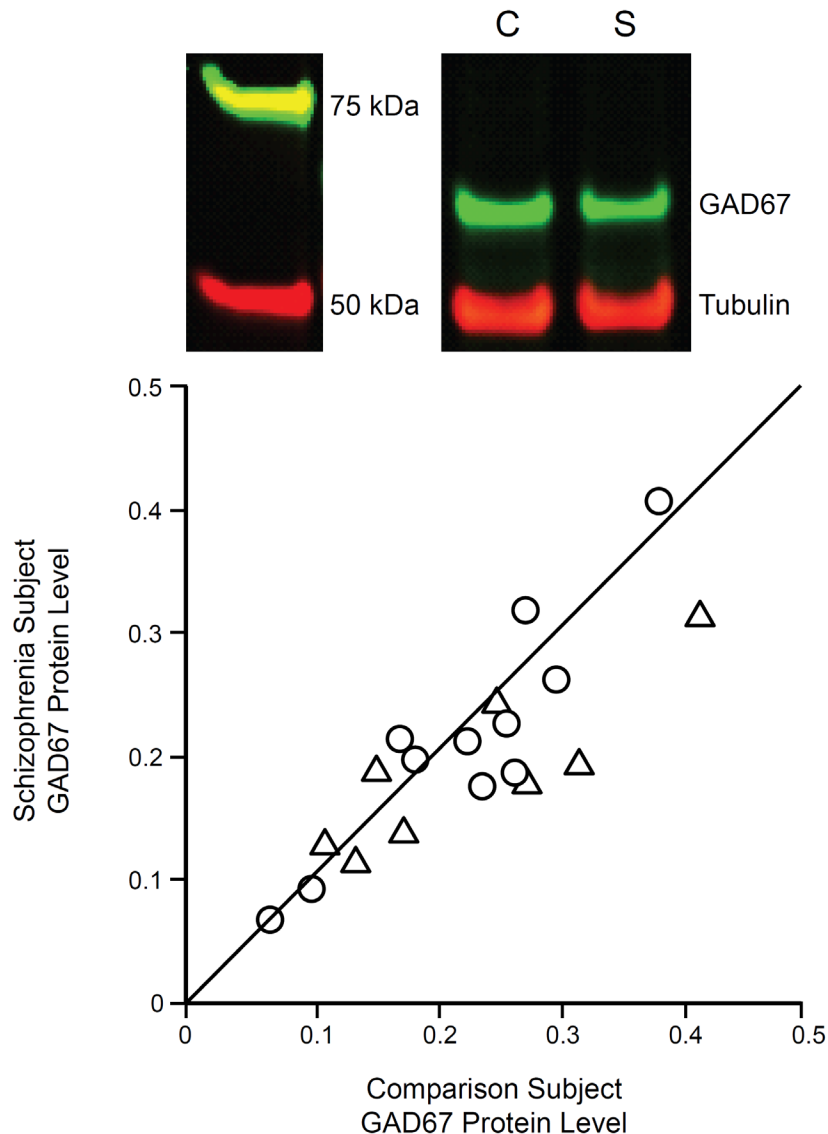
**Figure 15. Effect of substance abuse and psychotropic medications (top) and predictors of disease severity and measures of functional outcome (bottom) on GAD67 mRNA levels in schizophrenia subjects.** GAD67 mRNA levels in schizophrenia (circle) or schizoaffective (triangle) subjects did not differ as a function of the presence or absence of substance abuse or dependence ATOD; history of cannabis use; nicotine use ATOD; or antipsychotic, antidepressant, or benzodiazepine and/or valproic acid medications ATOD (all  $p \geq 0.486$ ) (top). Additionally, GAD67 mRNA levels did not significantly differ based on the presence or absence of predictors of disease severity (sex, schizophrenia vs. schizoaffective diagnosis, family history of first-degree relative with schizophrenia, or age of onset of illness) or measures of functional impairment (history of marriage, highest achieved socioeconomic status (SES), independent living, or death by suicide) (all  $p \geq 0.214$ ). Abbreviations: M, male; F, female; SZ, schizophrenia; SA, schizoaffective disorder; SES, socioeconomic status; DX, diagnosis; Benzo, benzodiazepine; VPA, valproic acid. Numbers at the bottom of bars indicate number of schizophrenia subjects per group. \*Information was not available for all subjects.

### 3.4.2 Total GAD67 protein levels in schizophrenia

Protein degradation begins upon death, but the effect of PMI on protein levels is variable across proteins and brain regions (Beneyto et al., 2009). Consequently, we examined the effect of PMI on GAD67 protein levels in the DLPFC from 6 comparison subjects as well as 1 monkey (See **2.3.2**). Based on these findings, we only used subject pairs with PMIs < 20 hrs for Western blot studies.

Uncorrected and tubulin-corrected GAD67 protein levels were significantly 12.9% ( $F_{1,17}=5.6$ ,  $p=0.015$ ) and 10.1% ( $F_{1,17}=3.4$ ,  $p=0.040$ ) lower in the schizophrenia subjects (**Figure 16**). In contrast, uncorrected tubulin levels did not differ between schizophrenia subjects and comparison subjects (-3.4%;  $F_{1,17}=2.3$ ,  $p=0.151$ ). Tissue storage time was not significant in either the uncorrected or corrected model (both  $F_{1,17} \leq 3.4$ ,  $p \geq 0.083$ ).

As depicted by the scatter along the unity line in **Figure 16**, GAD67 protein levels varied substantially across subject pairs. This scatter was over 4 times greater than for GAD67 mRNA levels (**Figure 14**), suggesting that it represented measurement variance. Consequently, we performed an additional 16 replicates in 4 subject pairs. The absolute levels of GAD67 protein differed between the first and second measurement in the same subject, but the percent difference within each subject pair was highly consistent (intra-class correlation coefficient=0.97). Together, these findings indicate that measurement variance contributes substantially to the distribution of GAD67 protein levels along the unity line in **Figure 16**, but the distance from the unity line for each subject pair likely reflects the biological effect of schizophrenia.



**Figure 16. GAD67 protein levels are significantly lower in schizophrenia subjects relative to comparison subjects.** The top image shows lanes from a representative blot loaded with tissue from a comparison and schizophrenia subject pair. The bottom plot shows tubulin-normalized GAD67 protein values for each pair of comparison and schizophrenia (circle) or schizoaffective (triangle) subjects. Values below the diagonal unity line indicate lower GAD67 protein in the schizophrenia subject relative to the comparison subject. GAD67 protein levels were significantly ( $F_{1,17}=3.4$ ,  $p=0.040$ ) 10.1% lower in schizophrenia subjects. Abbreviations: C, Comparison; S, Schizophrenia.

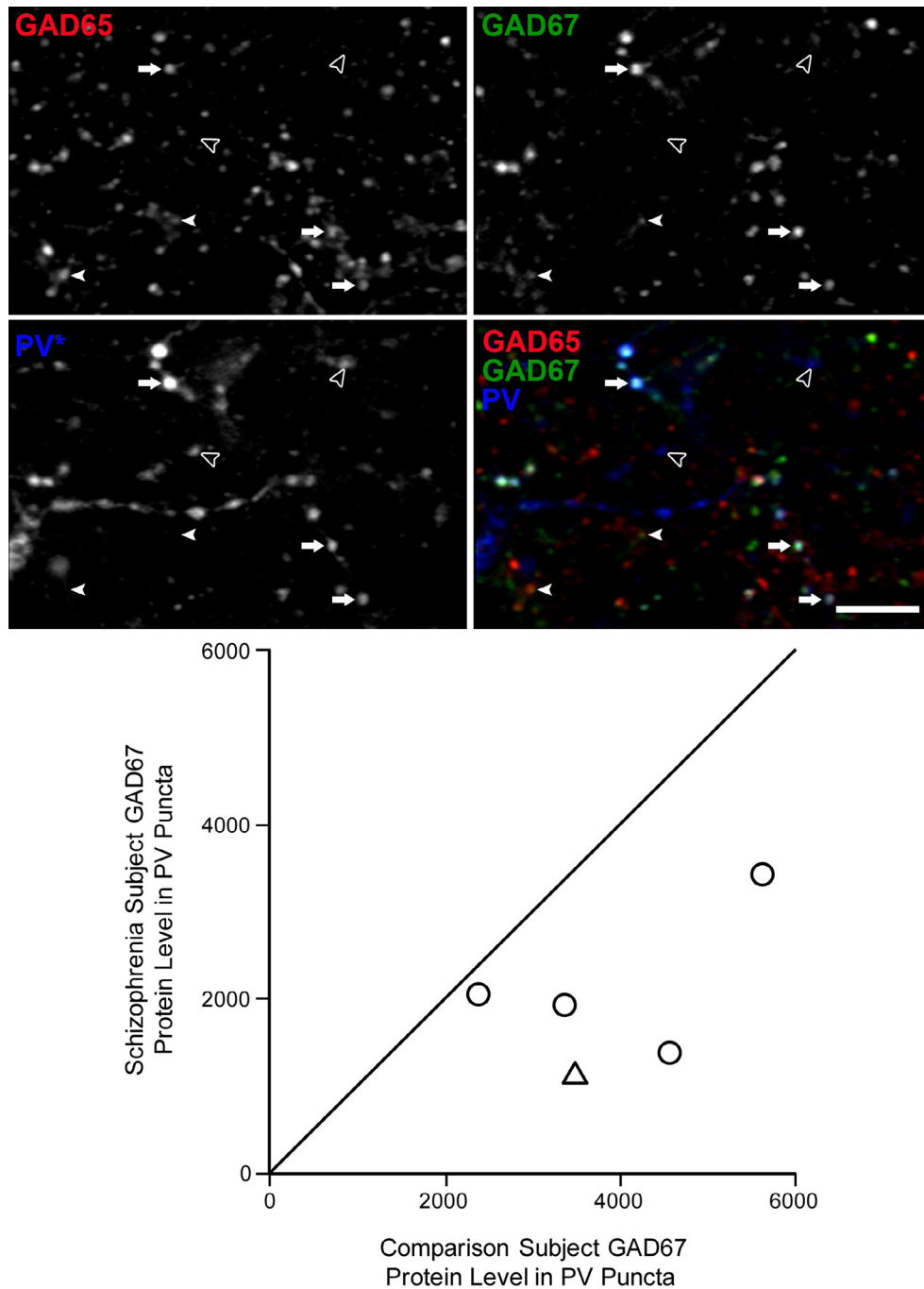
### 3.4.3 Correlation of total GAD67 mRNA and protein levels

Due to a lack of tissue availability, different comparison subjects were utilized between the total mRNA and protein studies for subject pair 4 (**Appendix B**). In the remaining 18 pairs of subjects, the within-pair percent differences in GAD67 mRNA and protein were positively correlated ( $r=0.40$ ;  $p=0.105$ ), supporting the idea that a reduction in GAD67 mRNA expression results in less translation of the cognate protein.

### 3.4.4 GAD67 protein levels in PV puncta

The deficit in GAD67 mRNA is known to be present in a subset of interneurons, including those that express PV (Hashimoto et al., 2003). Thus, we conducted a proof-of-concept test of the hypothesis that the deficit in GAD67 protein levels is much larger in the axon terminals of PV cells than in total cortical gray matter. Five subject pairs with a range of differences in total GAD67 protein levels (-24% to +16%; mean, -5%) were selected. We created masks of GAD65-IR puncta, with each mask object representing a putative axon terminal. In a total of 30,630 GAD65+/PV+ puncta, we measured GAD67 fluorescence intensity.

The fluorescence intensity of GAD67 in PV puncta was 48.8% lower ( $t_4=3.9$ ,  $p=0.008$ ) in schizophrenia subjects (**Figure 17**), a difference nearly 10X greater than the 5% reduction in total GAD67 protein found in these same subject pairs by Western blotting. These data suggest that GAD67 protein deficits are especially pronounced in PV neurons, consistent with prior findings of cell type-specific deficits in GAD67 mRNA (Hashimoto et al., 2003).



**Figure 17. GAD67 protein levels in PV puncta are significantly lower in schizophrenia.** The image projections (consisting of 3 z-planes) in the top panel show representative puncta obtained in the GAD65 (upper left), GAD67 (upper right), and PV (bottom left) channels. The bottom right image shows a color overlay of all 3 channels. Arrows indicate GAD65+/GAD67+/PV+ puncta; filled arrowheads indicate GAD65+/GAD67+/PV- puncta; open arrowheads indicate GAD65-/GAD67-/PV+ puncta.



Scale bar=10  $\mu\text{m}$ . \*For presentation purposes, pixel values were increased equally in order to visualize low, medium, and high intensity puncta in the PV channel, resulting in the saturation of some pixels. The scatter plot on the bottom shows GAD67 protein values in PV puncta plotted for each pair of comparison and schizophrenia (circle) or schizoaffective (triangle) subjects. Values below the diagonal unity line indicate lower GAD67 protein in PV puncta in the schizophrenia subject relative to the comparison subject. Mean GAD67 protein levels in PV puncta were significantly ( $t_4=3.9$ ,  $p=0.008$ ) 48.8% lower in schizophrenia subjects.

### 3.5 DISCUSSION

The results of this study document lower mean GAD67 mRNA levels in the DLPFC of schizophrenia subjects in the largest cohort reported to date. In a subset of this cohort, where potential confounds of protein measures were controlled, total GAD67 protein levels were also similarly lower, consistent with the hypothesis that lower mRNA levels lead to less translated protein. Finally, the GAD67 protein findings were especially pronounced in the axon terminals of PV neurons, providing evidence of a cell type-specific deficit in GAD67 protein at the site of GABA synthesis and release.

As in prior studies of GAD67 mRNA (Gonzalez-Burgos et al., 2010), the apparent magnitude of the GAD67 mRNA and protein deficits in the present study differed substantially across schizophrenia subjects. This variability does not appear to be due to differences across schizophrenia subjects in potential confounding factors such as substance abuse or psychotropic medication use ATOD. The similar levels of GAD67 mRNA in subjects both on and off antipsychotic medications ATOD are consistent with both prior studies of schizophrenia (Lewis et al., 2005) and the absence of a deficit in GAD67 mRNA levels in monkeys treated long-term with high-dose haloperidol decanoate (Volk et al., 2000), or low-dose oral haloperidol or olanzapine (Hashimoto et al., 2008a).

The variability in GAD67 mRNA levels across schizophrenia subjects also does not appear to be accounted for by factors that predict a more severe form of the illness, including male sex, a diagnosis of “pure” schizophrenia, a family history of schizophrenia, or an earlier age of onset. Additionally, factors that are a more direct metric of disease severity and functional outcome (death by suicide, lower SES, not living independently, and no history of marriage) were not associated with lower levels of GAD67 mRNA schizophrenia subjects. These data indicate that the GAD67 mRNA deficit is a conserved feature associated with a diagnosis of schizophrenia that is robust to the influence of multiple other factors.

Since GAD67 expression is activity-regulated (Benson et al., 1994), it is possible that lower GAD67 mRNA in schizophrenia is a consequence of reduced cortical activity associated with having a chronic psychiatric illness or severe cognitive impairments. However, the latter explanations seem unlikely since 1) GAD67 mRNA levels did not differ between schizophrenia subjects with or without factors that predict or reflect a more severe illness course, and 2) the within-pair percent differences in GAD67 mRNA and protein levels were not significantly correlated with length of illness ( $r=0.31$ ,  $p=0.134$  and  $r=-0.13$ ,  $p=0.605$ , respectively). Furthermore, lower frontal  $\gamma$  power, predicted to result from less GABA synthesis in PV neurons (Gonzalez-Burgos and Lewis, 2008; Cardin et al., 2009), is present during both the first episode (Minzenberg et al., 2010) and chronic (Cho et al., 2006) phases of schizophrenia. Taken together, these findings suggest that lower GAD67 mRNA is not attributable to substance abuse or psychotropic medication use, other factors related to illness course, or a consequence of having a chronic psychiatric illness. Thus, lower GAD67 levels appear to be a common component of, and not a consequence of, the disease process of schizophrenia.

The substantially greater deficit of GAD67 protein in PV puncta than in total gray matter is convergent with prior findings that the deficit in GAD67 mRNA expression is prominent in PV neurons (Hashimoto et al., 2003) and not detectable in the majority of other GABA neurons (Akbarian et al., 1995a; Volk et al., 2000). Lower levels of GAD67 protein in PV terminals suggest that these terminals synthesize less GABA, and therefore are less able to provide the inhibition of pyramidal neurons that is required for

$\gamma$  oscillations (Gonzalez-Burgos and Lewis, 2008; Cardin et al., 2009). Thus, impaired GABA synthesis in PV terminals, resulting in lower inhibition, is a plausible mechanism for the reduced prefrontal  $\gamma$  oscillation power observed during cognitive control tasks in subjects with schizophrenia (Cho et al., 2006; Minzenberg et al., 2010).

However, directly demonstrating that lower GAD67 mRNA and protein in the DLPFC [or other cortical regions where lower GAD67 mRNA has been observed (Gonzalez-Burgos et al., 2010)] lead to less cortical GABA in schizophrenia is challenging. Indeed, it is possible that GAD67 expression could be down-regulated in response to reduced GABA metabolism; for example, pharmacological inhibition of GABA transaminase, which metabolizes GABA, is associated with elevated cortical GABA and less GAD67 protein (Mason et al., 2001). Perhaps consistent with this interpretation, a magnetic resonance spectroscopy study reported elevated GABA levels in the anterior cingulate and parieto-occipital cortices of subjects with chronic schizophrenia (Ongur et al., 2010). However, some studies found no differences and others reported lower levels of cortical GABA in subjects with schizophrenia (Goto et al., 2009b; Yoon et al., 2010). Interestingly, lower GABA levels in the visual cortex in subjects with schizophrenia were correlated with reductions in a behavioral measure of visual inhibition that is known to depend on GABA neurotransmission (Yoon et al., 2010). Similarly, a trend-level negative correlation was found between frontal GABA levels and number of errors on a working memory task in patients with early-stage schizophrenia (Goto et al., 2009a), consistent with the idea that lower GABA synthesis results in cognitive impairments. However, since spectroscopic studies measure total tissue GABA levels, and not GABA levels in synaptic vesicles or the extracellular space, their relevance to GABA synthesis and transmission remain uncertain. The hypothesis that GABA hypofunction results in cognitive impairments in schizophrenia is supported by findings of impaired working memory performance in rats and monkeys after pharmacological blockade of prefrontal GABA<sub>A</sub> receptors (Enomoto et al., 2011; Sawaguchi et al., 1989).

In concert, the findings of the present study demonstrate that lower levels of GAD67 mRNA are accompanied by less GAD67 protein, which is especially pronounced in the axon terminals of PV GABA neurons, providing a plausible

mechanistic basis for impaired cortical  $\gamma$  oscillations and cognitive control deficits in schizophrenia. Importantly, comparisons across the subjects with schizophrenia suggest that a deficit in GABA synthesis is likely to contribute to cognitive dysfunction in schizophrenia and not to be the consequence of experiencing the illness.

#### **4.0    ROLE OF GAD67 IN REGULATING PARVALBUMIN AND GABA MEMBRANE TRANSPORTER 1 MRNA: IMPLICATIONS FOR SCHIZOPHRENIA**

**Modified from:** Curley AA, Eggan SM, Lazarus MS, Huang ZJ, Lewis DA. Role of GAD67 in regulating parvalbumin and GABA membrane transporter 1 mRNA: Implications for schizophrenia. In preparation.

##### **4.1    ABSTRACT**

Alterations in markers of GABA neurotransmission are present in the dorsolateral prefrontal cortex (DLPFC) of individuals with schizophrenia. Glutamic acid decarboxylase 67 (GAD67) mRNA and protein levels are lower, and are accompanied by lower levels of GABA membrane transporter 1 (GAT1) mRNA and protein. These alterations are thought to be most prominent in the parvalbumin (PV)-containing subclass of interneurons, which also contain lower levels of PV mRNA. Since PV and GAT1 can each reduce GABA levels available to postsynaptic receptors, it has been hypothesized that their reduction in schizophrenia may be a compensation to counteract upstream reductions in GAD67. However, it is not possible to directly test this hypothesis in schizophrenia. Thus, we characterized PV and GAT1 mRNA levels in two mouse models with lower GAD67. One model contains lower GAD67 specifically in PV neurons ( $PV^{GAD67+/-}$ ), while the other contains lower GAD67 in all interneurons ( $GABA^{GAD67+/-}$ ). Contrary to our hypothesis, neither PV nor GAT1 mRNA were altered at the tissue level in adult or developmental  $PV^{GAD67+/-}$  mice. Furthermore, cellular analyses confirmed a lower level of GAD67, but not of PV, mRNA in these animals. Additionally, PV and GAT1 mRNAs were also unaltered in  $GABA^{GAD67+/-}$  mice. Taken

together, these data suggest that lower GAD67 levels do not result in changes in PV or GAT1, and therefore lower GAD67 is unlikely to be the cause of reduced PV and GAT1 mRNA in schizophrenia.

## 4.2 INTRODUCTION

Schizophrenia is associated with alterations in several markers of GABA neurotransmission in the DLPFC (Lewis et al., 2005), and other cortical regions (Hashimoto et al., 2008b). Both mRNA (see Gonzalez-Burgos, 2010 for review) and protein (3.4; Guidotti et al., 2000) levels of GAD67, the enzyme responsible for the majority of cortical GABA synthesis, are lower in the DLPFC of subjects with schizophrenia. Levels of GAT1 mRNA and protein, which removes GABA from the extracellular space, are also lower in schizophrenia (Hashimoto et al., 2008b; Volk et al., 2001; Ohnuma et al., 1999; Woo et al., 1998). These deficits in GABA synthesis and reuptake appear to be most prominent in the PV-containing subclass of interneurons (3.4.4; Woo et al., 1998; Hashimoto et al., 2003) that also contain lower levels of PV mRNA (Hashimoto et al., 2003).

Through different mechanisms, PV and GAT1 can each reduce the amount of GABA available at postsynaptic receptors and therefore their lower expression in schizophrenia might be a compensatory response to counteract insufficient GABA synthesis (Lewis et al., 2005). PV is a slow  $\text{Ca}^{2+}$  buffer that increases the rate at which residual  $\text{Ca}^{2+}$  transients decay in GABA nerve terminals after neurotransmitter release (Collin et al., 2005; Muller et al., 2007). Therefore, during repetitive firing, reductions in PV levels in schizophrenia would increase the amount of calcium available to facilitate neurotransmitter release, resulting in increased synaptic GABA levels (Lewis et al., 2005). In fact, mice deficient in PV show enhanced GABA release from fast-spiking neurons (Collin et al., 2005; Vreugdenhil et al., 2003).

GAT1, one of four high-affinity  $\text{Na}^+/\text{Cl}^-$  transporters that take up extracellular GABA, is the most abundant GABA transporter in the cortex (Conti et al., 1998). Consistent with a role for GAT1 in removing extracellular GABA from the synaptic

space, in the hippocampus of GAT1 KO mice  $^3$ [GABA] uptake is virtually absent, while tonic GABA<sub>A</sub> receptor-mediated current is substantially increased (Jensen et al., 2003). Blockade of GAT1 increases the duration of evoked IPSCs at inhibitory synapses (Overstreet and Westbrook, 2003), suggesting that reducing GAT1 in schizophrenia would maximize the effect of the lower GABA levels (Lewis et al., 2005).

Although lower GAD67 in prefrontal PV neurons is well established in schizophrenia, whether this alteration produces the predicted downstream reductions in PV and GAT1 expression has not been directly tested in experimental models. Accordingly, in this study we measured developmental and adult levels of PV and GAT1 mRNAs in a mouse model with reduced GAD67 expression specifically in PV neurons, as well as model with lower GAD67 mRNA in all interneurons.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Animals

#### 4.3.1.1 PV-specific GAD67 Heterozygous (PV<sup>GAD67+/-</sup>) mice

PV<sup>cre/cre</sup> mice, in which cre expression is driven by the endogenous mouse Pv promoter, were produced as follows: A cassette containing the cre coding sequence, an internal ribosome entry site (IRES), and a polyadenylation site sequence (IRES-Cre-pA) was inserted into the 3' untranslated region of exon 5 of the endogenous Pv gene. Electroporation was used to insert the construct into 129/Ola ES cells and cells containing the construct were injected into blastocysts. The chimeric animals that resulted were crossed with C57BL/6 mice to yield PV<sup>cre/cre</sup> animals.

PV<sup>cre/cre</sup> mice were bred with Gad1<sup>lox/+</sup> mice (generated as described below) to produce litters containing mutant mice with one copy of the Gad1 gene removed in PV neurons (PV<sup>cre/+Gad1lox/+</sup>; subsequently referred to as PV<sup>GAD67+/-</sup> mice) and control mice (PV<sup>cre/+Gad1+/+</sup>; subsequently referred to as PV<sup>GAD67+/+</sup>). Animals were euthanized at 3 different ages: postnatal day 17 [**P17**; n=5 (2 males and 3 females) per genotype], P30 [n=6 (4 males and 2 females) per genotype], and P50 [n=6 (3 males and 3 females) per

genotype], and brains were removed, frozen, and stored at -80°C. Coronal sections from each brain were cut (12  $\mu$ m), thaw mounted onto SupraFrost slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C.

#### **4.3.1.2 Germ-line GAD67 Heterozygous (GABA<sup>GAD67+/-</sup>) mice**

As previously described (Chattopadhyaya et al., 2007), to generate GAD67 heterozygous (GABA<sup>GAD67+/-</sup>) mice, exon 2 (the first coding exon) of the Gad1 gene was flanked by loxP sites using gene targeting in ES cells. Using FLP recombinase, the Sv-NeoR selectable gene was removed to produce Gad1<sup>lox/+</sup> mice, which were interbred to generate phenotypically normal Gad1<sup>lox/lox</sup> mice. Gad1<sup>lox/lox</sup> mice were bred with Mox2-Cre mice to delete exon 2 in the germ-line (GABA<sup>GAD67+/-</sup>). Consistent with previously described GABA<sup>GAD67-/-</sup> mice (Asada et al., 1997), interbreeding of GABA<sup>GAD67+/-</sup> mice generated some animals that did not survive beyond the perinatal period. All animals were euthanized on P56 [n=7 (5 males and 2 females) per genotype]. Brains were processed and tissue was cut as described above.

#### **4.3.2 *In situ* hybridization**

ISH was performed as described previously (Hashimoto et al., 2005). A 311 base pair fragment corresponding to bases 151-461 of the mouse Gad1 gene (GAD67) (Genbank accession number Y12257), a 339 base pair fragment corresponding to bases 256-594 of the mouse Pvalb gene (PV) (GenBank accession number X59382), and a 462 base pair fragment corresponding to bases 691-1152 of the mouse Slc6a1 gene (GAT1) (GenBank accession number NM\_178703) were amplified by PCR. Nucleotide sequencing revealed 100% homology for the amplified fragments to previously reported sequences. Sense and antisense riboprobes were generated by *in vitro* transcription in the presence of <sup>35</sup>S-CTP using T7 or SP6 RNA polymerase, followed by digestion with DNaseI, and purified by centrifugation through spin columns. Following a standard hybridization protocol (Hashimoto et al. 2003), sections from all mice for a given comparison were exposed to BioMax MR film (Kodak, Rochester, NY) for 21 (GAT1), 40 (GAD67), or 72 (PV) hours.



For grain counting of GAD67 mRNA, slides were then coated with NTB2 emulsion (Kodak) using a mechanical dipper, Auto-dip Emulsion Coater (Ted Pella, Redding, CA), at a constant withdrawal speed and temperature, to ensure the consistency of emulsion thickness across sections. After 10 days, slides were exposed at 4°C, developed with D-19 (Kodak), washed, and counterstained using cresyl violet.

All slides for each comparison were contained in a single experimental run and exposed on the same film. Riboprobe specificity was confirmed by an absence of signal with sense probes and by the findings that each antisense probe produced the expected laminar pattern of mRNA expression (Hashimoto et al., 2005; Yasumi et al., 1997).

### 4.3.3 Quantification

To determine total GAD67, PV, and GAT1 mRNA levels, 3 evenly spaced coronal sections [spanning ~144 µm; +1.98 to +1.54 Bregma (Paxinos and Franklin, 2001)] containing primary and secondary motor (**M1** and **M2**) and medial prefrontal cortex (**mPFC**), including the cingulate and prelimbic cortices, from each animal were quantified as described previously (Hashimoto et al., 2005). Briefly, autoradiographic film images were captured and digitized using a Microcomputer Imaging Device (**MCID**) system (Imaging Research Inc., Canada) (5.1 µm/pixel resolution). All slides for each comparison were acquired in the same session with identical room illumination, black and gain levels, and flatfield correction. OD levels of GAD67, PV, and GAT1 were measured in each hemisphere from the pial surface to the white matter and expressed as nanoCuries per gram (**nCi/g**) of tissue by reference to <sup>14</sup>C standards (ARC, St. Louis, MO) exposed to the same film. All OD values were corrected by subtracting background measured in the white matter. In PV<sup>GAD67+/-</sup> mice, OD was measured within M1/M2, while in GABA<sup>GAD67+/-</sup> mice separate measures were made for M1/M2 and mPFC (see 4.4 for rationale). Within a region, measures from each hemisphere were averaged into a single measure per section.

To quantify the GAD67 and PV mRNA levels per neuron in the P50 PV<sup>GAD67+/-</sup> animals, emulsion-dipped and Nissl-counterstained sections were quantified as described previously (Hashimoto et al., 2005). MCID software and a Nikon microscope

(Melville, NY) with a motorized stage were used to place 2 (GAD67) or 4 (PV) immediately adjacent columns of sampling boxes (120  $\mu$ m x 170  $\mu$ m) continuously from the pial surface to the layer 6-white matter border in M1 of 2 of the 3 tissue sections utilized in the total GAD67 mRNA study. Sampling circles with a fixed diameter of 16  $\mu$ m were placed over GAD67 or PV silver grain clusters, and the number of silver grains per circle was quantified. All values were corrected by subtracting background grains per cluster measured in a 120  $\mu$ m x 170  $\mu$ m sampling box placed in the white matter. One pair of animals was not counted for PV due to a damaged slide. A total of 1630 and 1628 (GAD67) and 473 and 496 (PV) grain clusters were analyzed for PV<sup>GAD67+/+</sup> and PV<sup>GAD67+/-</sup> mice, respectively.

#### **4.3.4 Statistical analyses**

In order to control for experimental variance, each wild type animal was paired with an experimental animal, matched for sex and litter. Accordingly, paired t tests were used to test the effect of genetic condition on OD measures and number of grains per cluster using mean values across all of the sections from each animal. Analysis of variance (**ANOVA**) was used to assess the effect of age on OD in PV<sup>GAD67+/-</sup> animals. Since GAD67 levels were genetically manipulated to be lower in both models, all reported p values for GAD67 are one-tailed. All other p values are two-tailed.

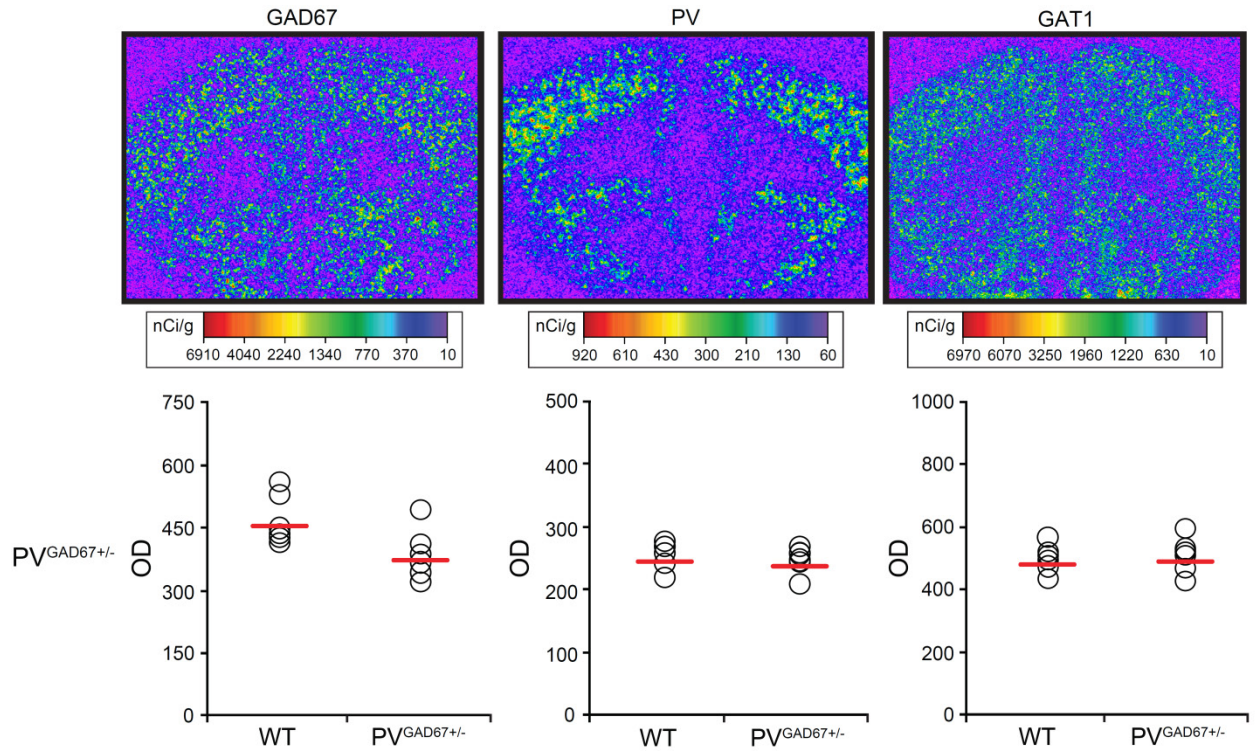
### **4.4 RESULTS**

#### **4.4.1 Transcript levels in PV<sup>GAD67+/-</sup> mice**

In addition to the DLPFC, lower levels of GAD67, PV, and GAT1 mRNAs in schizophrenia have been found in M1 (Hashimoto et al., 2008b). Since PV mRNA expression levels are low in the mouse mPFC (Thomsen et al., 2010; Hashimoto et al., 2005), likely due to the presence of significantly fewer PV neurons in mPFC than motor

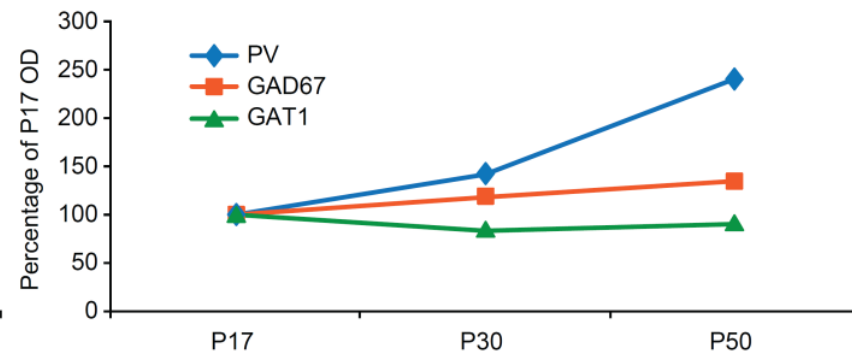
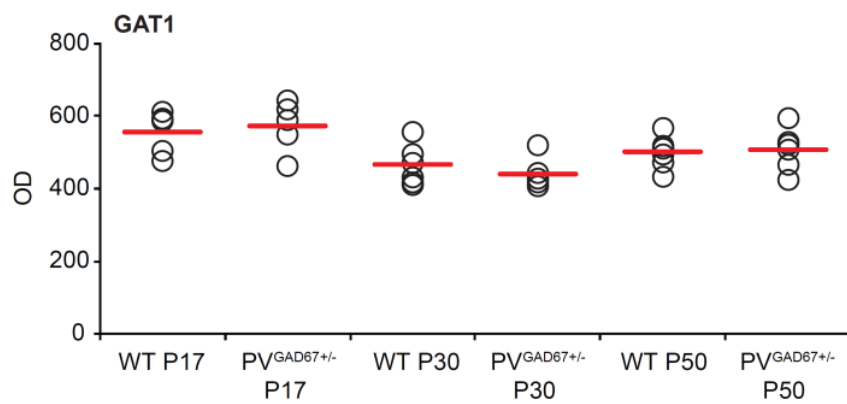
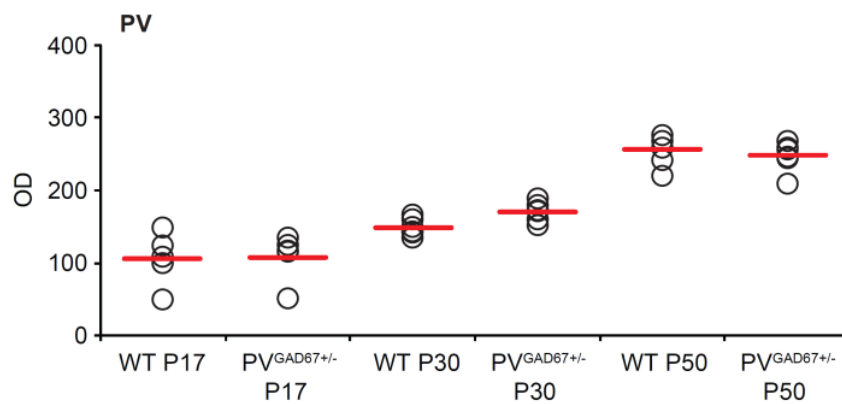
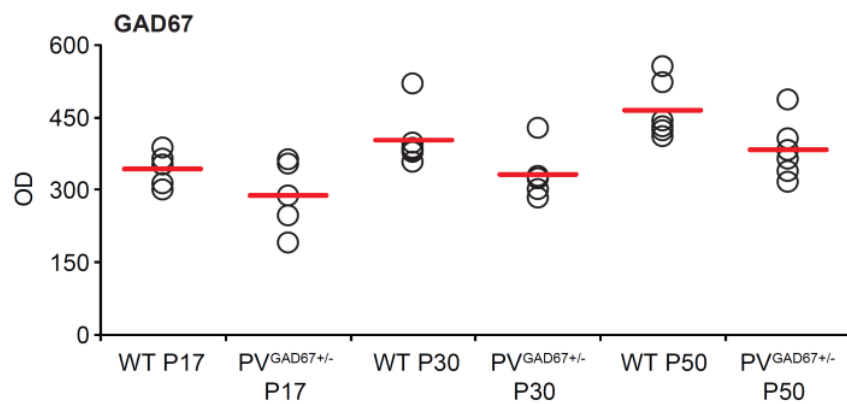
cortex (Fitzgerald et al., 2011), the latter may be a more sensitive region in which to detect changes in PV neurons. Maximizing sensitivity is especially important given that PV<sup>GAD67+/-</sup> mice exhibit lower GAD67 only in PV neurons. In fact, consistent with Fitzgerald et al. (2011), mean ( $\pm$ SD) GAD67 mRNA levels were only nonsignificantly 8.7% lower ( $t_5=1.18$ ,  $p=0.146$ ) in the mPFC, but significantly 17.4% lower ( $t_5=2.23$ ,  $p=0.038$ ) in M1/M2 of P50 PV<sup>GAD67+/-</sup> mice ( $384\pm60$ ) relative to PV<sup>GAD67+/+</sup> mice ( $465\pm60$ ) (**Figure 18**). Accordingly, we made all subsequent GAD67, PV, and GAT1 mRNA measures in M1/M2 in these animals.

Contrary to our hypothesis, neither PV ( $t_5=1.03$ ,  $p=0.350$ ) nor GAT1 ( $t_5=-0.31$ ,  $p=0.769$ ) mRNA levels significantly differed between PV<sup>GAD67+/-</sup> mice and PV<sup>GAD67+/+</sup> mice (**Figure 18**). In order to assess whether developmental compensations might account for the lack of change in PV and GAT1 in the P50 PV<sup>GAD67+/-</sup> mice, we examined 2 younger age groups of mice (P17 and P30). As shown in **Figure 19**, GAD67, PV, and GAT1 mRNAs all exhibited the predicted developmental trajectories in PV<sup>GAD67+/+</sup> mice (Kiser et al., 1998; de Lecea et al., 1995; Yan et al., 1997). Across development, GAD67 mRNA levels increased significantly by 135% ( $F_{2,14}=6.90$ ,  $p=0.01$ ) and PV mRNA increased significantly by 240% ( $F_{2,14}=55.85$ ,  $p=0.001$ ). In contrast, GAT1 decreased significantly by 10% ( $F_{2,14}=3.84$ ,  $p=0.047$ ) between P17 and P50. GAD67 mRNA levels exhibited similar magnitudes of reduction in PV<sup>GAD67+/-</sup> mice at both P17 and P30 as at P50 (**Figure 20**). In P17 animals, GAD67 mRNA was 15.8% lower in PV<sup>GAD67+/-</sup> mice ( $291\pm73$  nCi/g) relative to PV<sup>GAD67+/+</sup> mice ( $345\pm35$  nCi/g), though this difference only reached trend-level statistical significance ( $t_4=1.80$ ,  $p=0.074$ ). In P30 animals, GAD67 mRNA was significantly 17.7% lower ( $t_5=6.09$ ,  $p=0.001$ ) in PV<sup>GAD67+/-</sup> mice ( $334\pm50$  nCi/g) relative to PV<sup>GAD67+/+</sup> mice ( $406\pm49$  nCi/g).



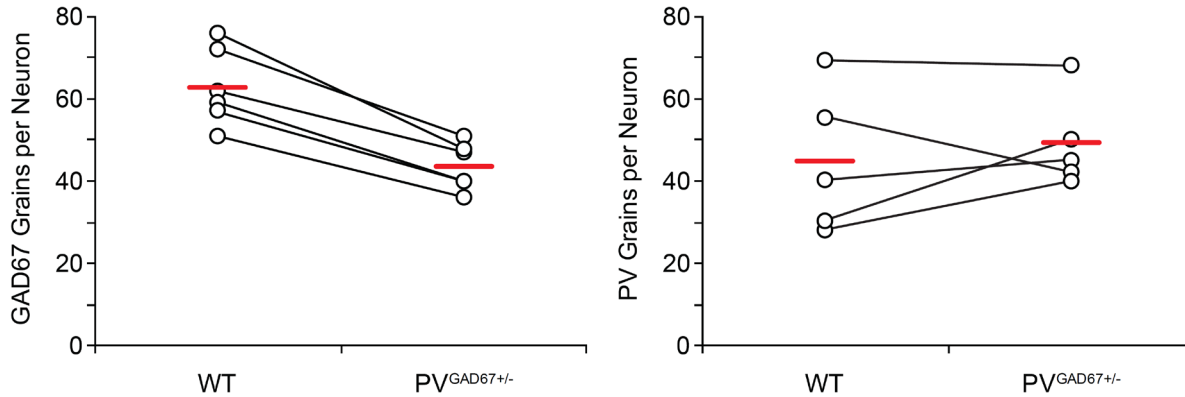
**Figure 18. GAD67, but not PV or GAT1, mRNA levels are reduced in PV<sup>GAD67+/-</sup> mice.** Top, representative film autoradiograms illustrating the expression of GAD67, PV, and GAT1 mRNA in M1/M2 of P50 PV<sup>GAD67+/-</sup> mice. The density of hybridization signal is presented in pseudocolor according to the calibration bars. Bottom, comparison of cortical GAD67, PV, and GAT1 mRNA levels by film OD in P50 PV<sup>GAD67+/-</sup> and PV<sup>GAD67+/+</sup> mice (GAD67:  $t_5=2.23$ ,  $p=0.038$ ; PV:  $t_5=1.03$ ,  $p=0.350$ ; GAT1:  $t_5=-0.31$ ,  $p=0.769$ ). Mean values for each group are indicated by the hash mark.

PV mRNA levels were not significantly different between PV<sup>GAD67+/-</sup> and PV<sup>GAD67+/+</sup> mice at P17 ( $t_4=-0.35$ ,  $p=0.740$ ), although there was a 13.9% increase in PV mRNA levels in PV<sup>GAD67+/-</sup> ( $171\pm13$  nCi/g) relative to PV<sup>GAD67+/+</sup> mice ( $150\pm12$  nCi/g) at P30 ( $t=-2.61$ ,  $p=0.048$ ) (**Figure 19**). GAT1 mRNA levels were not significantly different between PV<sup>GAD67+/-</sup> mice and PV<sup>GAD67+/+</sup> mice at either P17 ( $t_4=-0.42$ ,  $p=0.694$ ) or P30 ( $t_5=0.72$ ,  $p=0.510$ ) (**Figure 19**).



**Figure 19. GAD67, but not PV or GAT1, mRNA levels are reduced in PV<sup>GAD67+/-</sup> mice across development.** Comparison of GAD67, PV, and GAT1 mRNA levels by film OD in PV<sup>GAD67+/+</sup> and PV<sup>GAD67+/-</sup> mice (top left, top right, bottom left) (GAD67 P30:  $t_5=6.09$ ,  $p=0.001$ ; GAD67 P50:  $t_5=2.23$ ,  $p=0.038$ ; PV P30:  $t=-2.61$ ,  $p=0.048$ ; all others,  $p\geq 0.074$ ). Mean values for each condition are indicated by the hash mark. GAD67, PV, and GAT1 mRNA exhibit the expected developmental trajectories in M1/M2 of PV<sup>GAD67+/+</sup> mice (bottom right). GAD67 and PV mRNA increase, while GAT1 mRNA decreases, across development (GAD67:  $F_{2,14}=6.90$ ,  $p=0.01$ ; PV:  $F_{2,14}=55.85$ ,  $p=0.001$ ; GAT1:  $F_{2,14}=3.84$ ,  $p=0.047$ ).

PV neurons comprise approximately 50% of cortical interneurons in the mouse motor cortex (Tamamaki et al., 2003). Since PV<sup>GAD67+/-</sup> mice are predicted to have 50% lower GAD67 in all PV neurons, theoretically these animals should have approximately 25% less GAD67 mRNA than PV<sup>GAD67+/+</sup> mice. We performed a check of the genetic manipulation by quantifying GAD67 mRNA levels per neuron. The mean number of GAD67 grains per neuron was 30.5% lower ( $t_5=9.55$ ,  $p<0.001$ ) in PV<sup>GAD67+/-</sup> mice ( $44\pm 6$ ) relative to PV<sup>GAD67+/+</sup> mice ( $63\pm 9$ ) (**Figure 20**). These data confirm that the genetic manipulation did produce the expected reduction in GAD67. In contrast, the mean number of PV grains per neuron was not significantly different between PV<sup>GAD67+/-</sup> and PV<sup>GAD67+/+</sup> mice ( $t_4=-0.77$ ,  $p=0.483$ ) (**Figure 20**), suggesting that the lack of change in PV mRNA at the film level was not due to insufficient sensitivity. Since GAT1 is present in both PV cells as well as the non-PV cells that presumably contain normal levels of GAD67 mRNA, it would be difficult to detect a difference in only a subpopulation of GAT1 mRNA-containing cells. Thus, cellular levels of GAT1 mRNA were not quantified.



**Figure 20. Cellular levels of GAD67, but not PV, mRNA are reduced in PV<sup>GAD67+/-</sup> mice.** Comparison of M1 GAD67 (left) and PV (right) mRNA levels by grain counting in litter-matched pairs of P50 PV<sup>GAD67+/+</sup> and PV<sup>GAD67+/-</sup> mice (GAD67:  $t_5=9.55$ ,  $p<0.001$ ; PV:  $t_4=-0.77$ ,  $p=0.483$ ). Mean values for each condition are indicated by the hash mark.

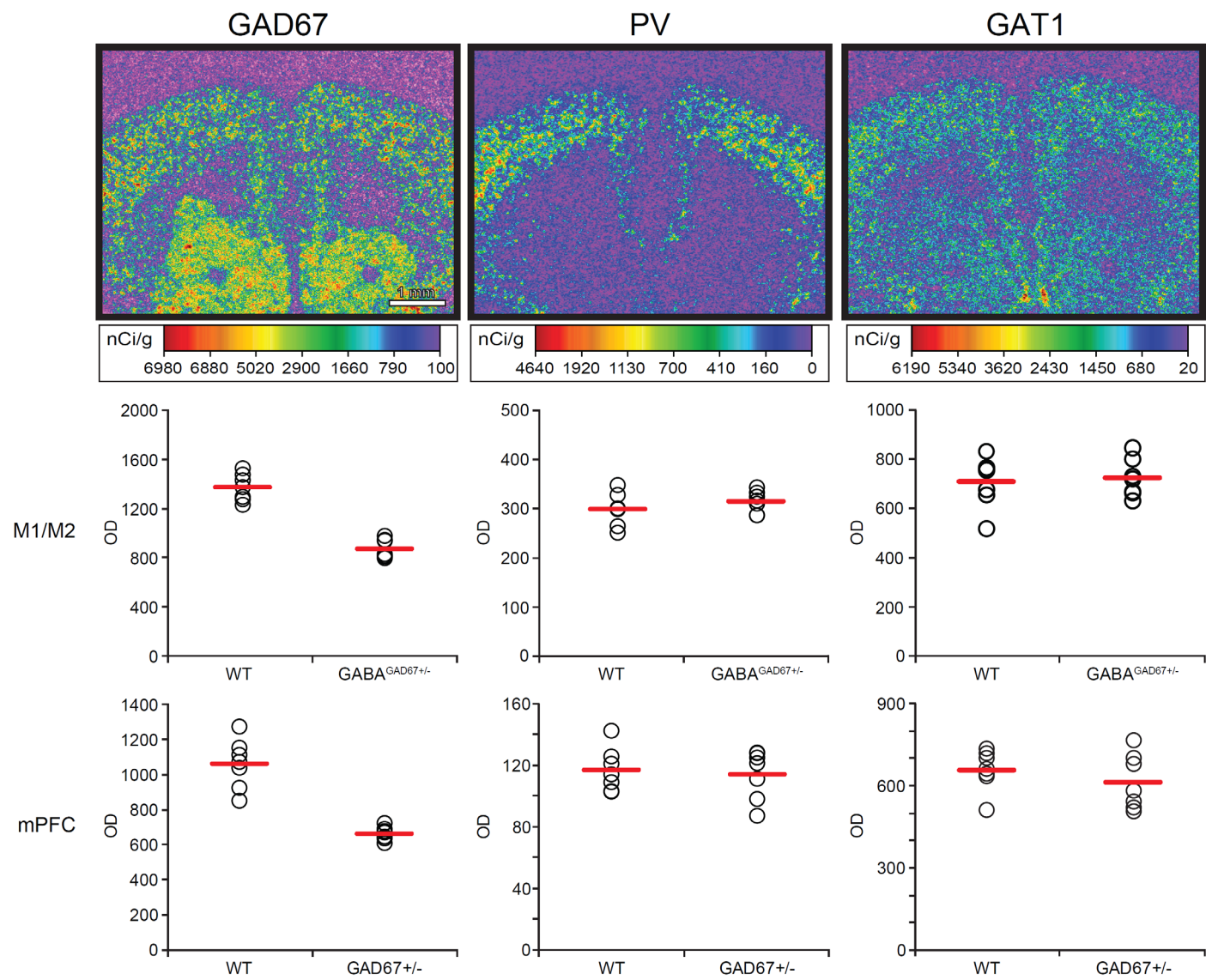
#### 4.4.2 Transcript levels in GABA<sup>GAD67+/-</sup> mice

In PV<sup>GAD67+/-</sup> mice, GAD67 is reduced specifically in PV neurons beginning on approximately P11, when PV mRNA expression turns on in the murine neocortex (del Rio et al., 1994). Given the lack of change in PV and GAT1 in PV<sup>GAD67+/-</sup> mice, we also examined these transcripts in a more pronounced and earlier model of GAD67 reduction, P56 germline GABA<sup>GAD67+/-</sup> mice, in which tissue levels of GAD67 mRNA are reduced in all interneurons in early prenatal development (Eggan et al., 2011). In M1/M2, GAD67 mRNA levels were significantly 37.2% lower ( $t_6=7.89$ ,  $p<0.001$ ) in GABA<sup>GAD67+/-</sup> ( $872\pm78$  nCi/g) compared to GABA<sup>GAD67+/+</sup> ( $1375\pm111$  nCi/g) mice (**Figure 21**). In contrast, neither PV ( $t_6=-1.03$ ,  $p=0.344$ ) nor GAT1 ( $t_6=-0.36$ ,  $p=0.731$ ) mRNA expression levels were significantly different between GABA<sup>GAD67+/+</sup> and GABA<sup>GAD67+/-</sup> mice in M1/M2 (**Figure 21**). In order to exclude the possibility that the lack of change in PV and GAT1 mRNA is region-specific, we also examined these transcripts in mPFC. Similar to the findings in M1/M2, no significant changes in PV ( $t_6=0.33$ ,  $p=0.754$ ) or



GAT1 ( $t_6=1.32$ ,  $p=0.235$ ) mRNA were detected in the mPFC of GABA<sup>GAD67+/-</sup> mice relative to GABA<sup>GAD67+/+</sup> mice (**Figure 21**).





**Figure 21. GAD67, but not PV or GAT1, mRNA levels are reduced in GABA<sup>GAD67+/-</sup> mice.** Top, representative film autoradiograms illustrating the expression of GAD67, PV, and GAT1 mRNA in P56 GABA<sup>GAD67+/+</sup> mice. The density of hybridization signal is presented in pseudocolor according to the calibration bars. Middle, comparison of M1/M2 and mPFC GAD67, PV, and GAT1 mRNA levels by film OD in P56 GABA<sup>GAD67+/+</sup> and GABA<sup>GAD67+/-</sup> mice (GAD67:  $t_6=7.89$ ,  $p<0.001$ ; all others:  $p\geq 0.344$ ). Bottom, comparison of mPFC GAD67, PV, and GAT1 mRNA levels by film OD of P56 GABA<sup>GAD67+/+</sup> and GABA<sup>GAD67+/-</sup> mice [GAD67:  $t_6=7.67$ ,  $p<0.001$  (data from Eggen et al., 2011); all others:  $p\geq 0.235$ ]. Mean values for each group are indicated by the hash mark.

## 4.5 DISCUSSION

In PV<sup>GAD67+/-</sup> mice, GAD67 mRNA was significantly reduced by ~17% at the tissue level across development and by 31% at the cellular level in adult animals. Tissue-level GAD67 mRNA levels were significantly reduced by 37% in adult GABA<sup>GAD67+/-</sup> mice. However, PV and GAT1 mRNA levels were not significantly altered in either mouse model at the tissue level. Moreover, PV was not significantly altered at the cellular level in PV<sup>GAD67+/-</sup> mice. Thus, reduced GAD67 expression does not affect the levels of PV and GAT1 mRNAs, regardless of cell type-specificity or developmental age of the deficit in GAD67 mRNA expression. These data do not support the hypothesis that PV and GAT1 are down-regulated in schizophrenia as a consequence of lower GAD67 in PV neurons.

It is possible that compensatory increases in other gene products, such as GAD65, or in protein levels of GAD67, may result in normal GABA levels in these animals. However, in visual cortex P30 PV<sup>GAD67+/-</sup> mice exhibit approximately 30% less GAD67 immunoreactivity in PV neurons, as well as several functional impairments (Lazarus and Huang, 2010). Significantly reduced IPSC amplitude and significantly faster IPSC decay time, as well as pyramidal cell hyperactivity have been observed, all

of which are consistent with lower GABA function (Lazarus and Huang, 2010). Although differences between motor and visual cortices may exist, both regions exhibit lower GAD67 mRNA in schizophrenia (Hashimoto et al., 2008b), and therefore these data suggest that the lack of change in PV and GAT1 mRNAs in PV<sup>GAD67+/-</sup> mice is not due to normal GABA levels resulting from compensatory changes in other effectors of GABA neurotransmission.

Based on the 17% reduction in GAD67 mRNA in PV<sup>GAD67+/-</sup> mice, which is comparable to the mean 15% reduction in GAD67 mRNA in schizophrenia (**3.4.1**), these animals capture the average difference observed in schizophrenia. This suggests that the lack of change in PV and GAT1 mRNA is not because these mouse models exhibit a level of GAD67 reduction that is insufficient to model the changes in schizophrenia. However, the lack of PV and GAT1 mRNA changes could be due to an inappropriate modeling of the time course of the reduction of GAD67 in schizophrenia.

Both genes and the environment are thought to play a role in the pathogenesis of schizophrenia; lower GAD67 resulting from genetic factors would likely occur at the onset of prenatal development, whereas an environmental insult, such as complications during late pregnancy, would produce a deficit later (Hoftman and Lewis, 2011). Here we have modeled these two possible time points of lower GAD67 expression. GABA<sup>GAD67+/-</sup> mice model a reduction in GAD67 early in prenatal development, whereas PV<sup>GAD67+/-</sup> mice do not exhibit lower GAD67 until later in development [P11, corresponding to roughly the third trimester of gestation in humans (Clancy et al., 2001)]. Our data suggest that PV and GAT1 mRNAs are not reduced regardless of whether the GAD67 deficit is present from the birth of GABA neurons or not until later in development.

However, it is currently unclear when GAD67 is reduced in schizophrenia. Allelic variations in the GAD1 gene are associated with schizophrenia, and with lower GAD67 mRNA (Straub et al., 2007), suggesting that GAD67 may in fact be reduced early in prenatal development. On the other hand, the small effect size and low penetrance suggests that genetic variations of GAD1 may only play a role in the pathogenesis of schizophrenia in a minority of cases (Insel, 2010). Additionally, an argument against the role of prenatal risk factors in the etiology of schizophrenia has recently been made,

citing the lack of reproducibility of specific factors (van Os et al., 2010). Thus, it is also possible that GAD67 is not reduced until a postnatal time point in schizophrenia. In fact, in children who go on to develop schizophrenia as adults, working memory performance is normal at 7 years of age (Reichenberg et al., 2010). By ages 9-13, however, their performance lags behind that of their peers (Reichenberg et al., 2010), suggestive of a deficit in DLFPC circuitry that does not manifest until later in childhood development. Thus, reducing GAD67 very early in development, as we have done in the present study, may invoke different effects than a reduction during childhood in individuals with schizophrenia would.

PV development occurs postnatally in rodents (de Lecea et al., 1995), but this time point corresponds to prenatal development in humans (Clancy et al., 2001). In contrast, PV development occurs postnatally in primates (reviewed in Hoftman and Lewis, 2011). Furthermore, there are clear differences in PV<sub>b</sub> cells between rodents and primates, with the latter being more excitable (Povysheva et al., 2008). Therefore, it is possible that reducing GAD67 in rodent and primate PV cells would produce different compensatory effects. Thus, our data do not support the hypothesis that PV and GAT1 mRNA are lower in schizophrenia as a compensation for lower GAD67 in PV cells, but clear species differences in PV neurons prevent the definitive rejection of this hypothesis.

Alternatively, it is possible that changes in PV or GAT1 may be upstream of lower GAD67 in schizophrenia. For example, lower levels of PV increase GABA release (Collin et al., 2005; Vreugdenhil et al., 2003) and the resulting increase in extracellular GABA might evoke a compensatory reduction in GABA synthesis via downregulation of GAD67 expression. Likewise, a deficit in GABA reuptake may result in increased GABA availability and a compensatory downregulation of GABA synthesis. However, GAT1 KO mice actually exhibit increased GAD67 (Bragina et al., 2008).

Although we cannot rule out that differences in the time point of the GAD67 reduction, or disparities between mouse and human PV neurons, may account for the differences between the mouse model and schizophrenia data, the findings of the present study suggest that PV and GAT1 are not reduced in the illness as a consequence of lower GAD67 in PV neurons. Thus, an alternative, upstream

mechanism may be responsible for concomitant changes in GAD67, PV, and GAT1. For example, reduced signaling through tyrosine kinase B (**TrkB**) receptors, which exhibit lower mRNA expression schizophrenia (Hashimoto et al., 2005; Weickert et al., 2005), produces alterations in GAD67 and PV mRNA (Hashimoto et al., 2005). Additionally, lower signaling through NMDA receptor 1 (**NR1**), also altered in schizophrenia (Lewis and Moghaddam, 2006), produces lower levels of GAD67, PV and GAT1 mRNA (Behrens et al., 2007; Bullock et al., 2009; Belforte et al., 2010). Future studies are needed to further assess the mechanisms underlying lower PV and GAT1 expression in schizophrenia.

## 5.0 GENERAL DISCUSSION

This dissertation assessed factors contributing to the heterogeneity of the GAD67 mRNA deficit in schizophrenia, overcame significant technical challenges to quantify both total and PV axon terminal levels of GAD67 protein in postmortem human brain, and examined the consequences of lower GAD67 expression in PV neurons on PV and GAT1 mRNA levels. Briefly, we found that 1) lower GAD67 mRNA in schizophrenia is not associated with medication or substance use, predictors or measures of functional outcome, or length of illness; 2) GAD67 protein is similarly reduced in the DLPFC; and 3) the GAD67 protein deficit is much greater at PV axon terminals, a major site of GABA production and function. Taken together, these data suggest that GABA synthesis is lower and consequently neurotransmission is impaired in a subset of cortical inhibitory neurons in schizophrenia. Additionally, we found that reducing GAD67 expression in PV neurons does not seem to alter PV or GAT1 expression. Thus, lower PV and GAT1 mRNAs in schizophrenia are likely not a consequence of lower GAD67.

The concluding section of **Chapter 1** raised several questions concerning the nature of the GAD67 deficit in schizophrenia. In the following sections, I will address these questions, as well as several that arise from our data, and consider important future directions for research. First, I will discuss how common the GAD67 deficit is in schizophrenia, and identify sources that may contribute to the observed variability **(5.1)**. Second, I will discuss the significance of lower total GAD67 protein levels in schizophrenia **(5.2)**. Third, I will consider the implications of lower GAD67 protein levels at PV axon terminals **(5.3)**. Fourth, I will review several potential mechanisms that may underlie lower GAD67 expression in PV neurons, and discuss the time point in the disease process when the deficit in GAD67 occurs **(5.4)**. Lastly, I will examine the consequences of lower GAD67 in PV neurons **(5.5)**.

## 5.1 HOW COMMON IS THE GAD67 MRNA DEFICIT, AND WHAT FACTORS CONTRIBUTE TO THE HETEROGENEITY?

Schizophrenia is a disease characterized almost as well by its heterogeneity as by its common features. A diagnosis is often difficult due to the fact that it is made based solely on clinical features. Additionally, schizophrenia has a very broad presentation that includes several diagnostic subtypes. Finally, outcomes vary widely; a minority of patients experience full symptom remission, while others are severely disabled for the rest of their lives (Harrison et al., 2001; Insel, 2010). Throughout the literature, the GAD67 mRNA deficit is also characterized by substantial heterogeneity. Relative to comparison subjects, most patients exhibit lower levels of GAD67, while some exhibit normal, and even increased, levels of the enzyme (Volk et al., 2000; Hashimoto et al., 2005; Hashimoto et al., 2008b).

### 5.1.1 Proportion of schizophrenia subjects with lower GAD67 mRNA

In the 13 published studies that have examined GAD67 mRNA expression in the DLPFC in schizophrenia to date, 5 provided data on the number of subject pairs with lower GAD67 mRNA in the schizophrenia subject (**Table 2**). Although limited by overlapping cohorts, averaging the results from each study in **Table 2** together suggests that roughly 87% of schizophrenia subjects contain lower GAD67 mRNA. In the present study, we also observed heterogeneity in GAD67 mRNA levels (**Figure 14**). In 31 of the 42 subject pairs (74%) the schizophrenia subject exhibited less GAD67 mRNA than the matched comparison subject.

**Table 2. GAD67 mRNA studies utilized in analysis of GAD67 heterogeneity<sup>a</sup>**

Study	Pairs with lower GAD67 mRNA in the schizophrenia subject
Volk et al., 2000 (ISH)	8/10; 9/10; 8/10 depending on layer
Hashimoto et al., 2005 (ISH)	15/15 in cohort 1; 9/12 in cohort 2
Huang and Akbarian, 2007 (qPCR)	14/14; 13/14; 12/14 depending on primers
Hashimoto et al., 2008a (qPCR, microarray)	10/14 by both qPCR and microarray
Hashimoto et al., 2008b (qPCR)	12/12

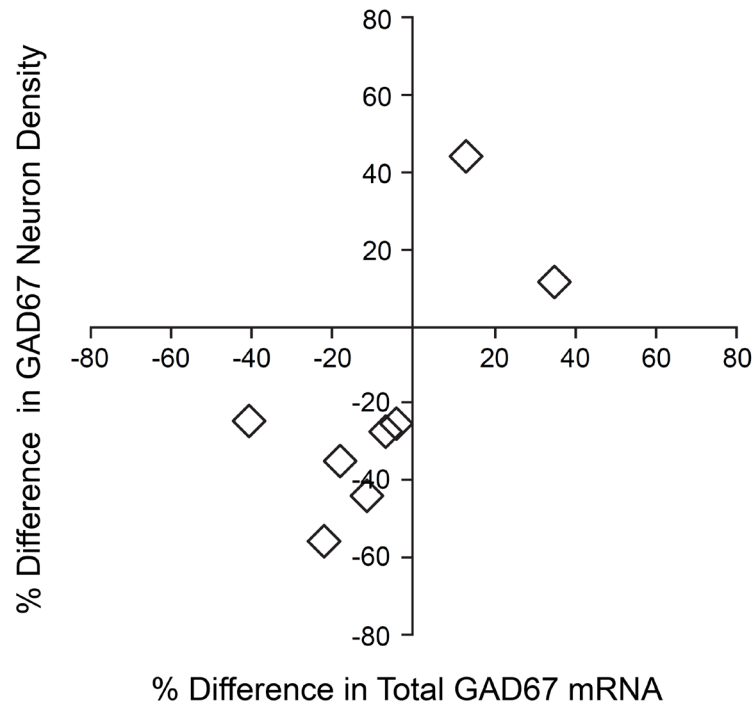
<sup>a</sup>Abbreviations: ISH, *in situ* hybridization.

One advantage of using paired comparisons to assess the commonality of the GAD67 mRNA deficit is that this approach controls for the potential influence of pairing variables, such as sex and age, on GAD67 mRNA levels. However, paired comparisons are influenced by the variability of not only the schizophrenia subject but also the comparison subject. A second strategy that is more robust to comparison subject variability, though potentially confounded by differences in pairing variables, is to relate each schizophrenia subject to the mean of the comparison group. When utilizing this method in two of the studies from **Table 2**, 24 out of 27 subjects, assessed using ISH (89%; Hashimoto et al., 2005), and 10 out of 12 subjects, using qPCR (83%; Hashimoto et al., 2008b), exhibit lower GAD67 mRNA. When comparing schizophrenia subjects to the mean of comparison subjects in the current study, 30 of the 42 subjects (71%) exhibit lower GAD67 mRNA. The differences in GAD67 mRNA observed using this approach are quite similar to those observed using paired comparisons, suggesting that neither variability due to comparison subjects or pairing variables substantially confounds the measures of commonality.

One limitation of the above analyses is that all differences in GAD67 mRNA, however small, are counted as biologically meaningful. In fact, a 5% reduction may not



be indicative of a functionally meaningful deficit. For example, Parkinson's disease symptoms do not typically begin until 50-70% of dopamine cells have been lost in the substantia nigra (Halliday and McCann, 2010), and thus a 30% cell loss may not be clinically meaningful. However, determining the criterion for a meaningful difference in schizophrenia is difficult. One approach to this issue is to make a within-pair comparison of total GAD67 mRNA levels and GAD67 mRNA+ neuron density, the latter being an indicator of the proportion of GAD67 cells that drop below the threshold of detectability of grain counting, and thus exhibit marked reductions in GAD67. In fact, such data are available for the 8 subjects pairs in common between the qPCR study presented here **(3.4.1)** and the grain counting study by Volk et al. (2000). As observed in **Figure 22**, even very modest deficits in GAD67 by qPCR are associated with more than 20% fewer cells with detectable GAD67 levels. This comparison allows for a determination of what magnitude of a tissue-level reduction is needed in order to observe a large reduction in cellular levels of GAD67 mRNA. Examination of the levels of GAD67 mRNA per neuron is a biologically meaningful measure since it evaluates the proportion of cells without detectable levels of the transcript, thereby indexing cells that are presumably very functionally impaired. In fact, in  $PV^{GAD67+/-}$  mice a 31% reduction in the GAD67 mRNA levels per neuron, but no loss in the number of neurons with detectable levels of GAD67 **(4.4.1)**, is able to produce functional impairments such as reduced IPSC amplitude (Lazarus and Huang, 2010). Thus, an even larger reduction, such as the one needed in order for some neurons to drop below detectability, is surely associated with functional deficits. These data suggest that even 5-10% decrements in total GAD67 mRNA in tissue homogenates may be biologically meaningful, since they indicate a marked decrease in GAD67 mRNA levels in a subset of neurons that produces functional impairment. Interestingly, when utilizing both total GAD67 mRNA and GAD67 neuronal density, 6 out of 8 pairs (75%) exhibit lower GAD67 in the subject with schizophrenia, which closely matches the above mean values observed across multiple studies.



**Figure 22. Pairwise percent differences in GAD67 neuronal density plotted against percent differences in total GAD67 mRNA.** GAD67 mRNA+ neuronal density was assessed using grain counting; total GAD67 mRNA was measured using qPCR.

### 5.1.2 Sources of GAD67 mRNA variability

Taken together, the results from the current study as well as from the literature suggest that approximately 75% of schizophrenia subjects do exhibit lower levels of cortical GAD67 mRNA. But what accounts for the proportion of patients that do not exhibit lower GAD67? Is the observed variability in GAD67 mRNA levels simply due to either methodological or biological variability that is inherent to human postmortem studies, or is it specific to schizophrenia? Methodological variability does not seem to play a large role in the heterogeneity among GAD67 mRNA levels, since different techniques converge on very similar results. For example, the within-pair percent changes in GAD67 mRNA assessed using qPCR and ISH are highly correlated ( $r=0.89$ ,  $p<0.001$ ) (Hashimoto et al., 2008a). Similarly, the same qPCR data are significantly correlated

with the expression differences detected by DNA microarray ( $r=0.71$ ,  $p=0.005$ ) (Hashimoto et al., 2008a). Additionally, as shown in **Figure 22**, assessments of both cellular and tissue levels of GAD67 in schizophrenia converge on the same disease effect. That is, across both measures, the directionality of the GAD67 change is identical for every pair.

Interestingly, in the present study, the GAD67 mRNA CV for the schizophrenia subjects (0.247) was double that of the comparison subjects (0.125), suggesting that the heterogeneity observed is due, at least in part, to factors that differentially affect schizophrenia and comparison subject brains. In fact, this phenomenon is also observed throughout the literature, with virtually all studies for which data are available exhibiting higher variability in schizophrenia subjects relative to comparison subjects (Akbarian et al., 1995a; Volk et al., 2000; Hashimoto et al., 2005; Hashimoto et al., 2008b; Hashimoto et al., 2008a; Woo et al., 2008). Thus, greater variability seems to be a conserved feature of schizophrenia, due to either the disease process itself or an associated factor.

Importantly, the observed variability does not seem to be accounted for by confounding factors such as use of antipsychotics, benzodiazepine/valproic acid, or antidepressants; or with alcohol abuse or dependence, nicotine use, or a history of marijuana use, since GAD67 mRNA levels were not significantly different in schizophrenia subjects with or without these factors (**Figure 15**). Moreover, GAD67 mRNA levels did not differ as a function of age or length of illness (see **5.4.2** for further discussion), suggesting that lower GAD67 mRNA in schizophrenia is not a consequence of having the illness. Importantly, the fact that the GAD67 alterations seem to be specific to the disease process of schizophrenia suggests that ameliorating these deficits may lead to an alleviation of cognitive control symptoms and improvement of functional outcome.

Given that lower GAD67 mRNA is thought to underlie the cognitive dysfunction in schizophrenia, and cognitive symptoms are the best predictor of functional outcome (Green et al., 2000), we hypothesized that GAD67 mRNA levels may be different between schizophrenia subjects as a function of predictors of illness severity and/or measures of functional outcome. This, however, was not observed, as there was no

change in GAD67 mRNA levels in schizophrenia subjects as a function of sex, a diagnosis of schizophrenia rather than schizoaffective disorder, a first degree relative with schizophrenia, an early age of onset, suicide, no history of marriage, low SES, or independent living (**Figure 15**).

It is possible that the above variables represent too crude of an assessment of illness severity and functional outcome in order to detect differences in GAD67 mRNA. For example, many healthy individuals never marry, and some schizoaffective disorder subjects experience a worse course of illness than some schizophrenia subjects. One approach that may increase the ability to detect a difference is to create a composite index of disease severity that incorporates multiple predictive factors into a single measure. To do this, we assigned each schizophrenia subject a +1 on each factor that was positively associated with a more severe illness course or more impaired functional outcome, and a -1 on each factor that was associated with a less severe illness course or less impaired functional outcome; a total was then made for each subject. Values ranged from -6 to +4; a more positive number is associated with greater illness severity. However, there was no significant correlation between composite index of severity and GAD67 mRNA levels ( $r=-0.045$ ,  $p=0.778$ ). One caveat from both the individual and composite measures is that, due to differences in subject age ATOD, each measure was assessed at different time points in the lifespan of individual subjects, which may influence some values obtained. Additionally, the measures are based on retrospective psychological autopsy records obtained from next of kin, and thus may be subject to inaccuracies. Ideally, though not available for the current cohort, data on more direct measures such as laboratory assessments of cognitive functioning would be the most informative. Future studies utilizing this type of information may provide insight into the sources of GAD67 heterogeneity.

The variability in the GAD67 deficit does not seem to be accounted for by different schizophrenia subtypes, evidenced by the fact that we did not observe a significant effect of diagnosis subtype (chronic, paranoid, undifferentiated, or schizoaffective disorder) on GAD67 mRNA levels ( $F_{1,38}=0.547$ ,  $p=0.653$ ). This is not particularly surprising given that the different subtypes are based on differences in positive and negative symptomatology, and not the cognitive impairments thought to

result from alterations in GAD67. Alternatively, it is possible that schizophrenia is caused by multiple mechanisms that converge on a common symptomatology, and thus the heterogeneity in GAD67 levels may be due to differing mechanisms between different subjects. For example, as described below in **5.3**, lower GAD67 mRNA can be achieved in several ways, and perhaps each etiopathogenic mechanism gives rise to a different level of GAD67 reduction in schizophrenia.

## **5.2 WHAT IS THE SIGNIFICANCE OF LOWER LEVELS OF TOTAL GAD67 PROTEIN IN SCHIZOPHRENIA?**

Total GAD67 protein levels are significantly 10% lower in the DLPFC of subjects with schizophrenia relative to normal comparison subjects (**Figure 16**). Furthermore, the reductions in mRNA and protein are similar in magnitude and tend to be correlated, suggesting that the reduction in GAD67 message drives the reduction in protein. Thus, the large numbers of studies replicating lower levels of GAD67 mRNA can be reasonably interpreted as extending to the protein level. Lower levels of the enzyme responsible for the majority of cortical GABA synthesis (Battaglioli et al., 2003; Mason et al., 2001) presumably result in lower cortical GABA levels in schizophrenia. However, as discussed in **3.5**, magnetic resonance spectroscopy studies of cortical GABA levels in schizophrenia have produced mixed results (Ongur et al., 2010; Goto et al., 2009b; Yoon et al., 2010). Given that spectroscopic studies measure total tissue GABA levels, not those associated with synapses, their relevance to GABA neurotransmission is unclear. Thus, future studies are needed to properly assess synaptic GABA levels in schizophrenia.

The significant 10% reduction in total GAD67 protein by Western blotting observed here differs from the 50% lower (Guidotti et al., 2000) and unchanged (Straub et al., 2007; Dracheva et al., 2004) levels reported in the literature. An effect of PMI may explain these differences. Although we found that GAD67 protein is relatively well-preserved at PMIs of up to 26 hours (with 82% of levels of 7 hr PMI group still present; **2.3.2**), tissue was not available with which to examine the effect of PMI at longer time

points. Given the substantially longer mean PMI of the schizophrenia group (34 hrs) than the normal comparison group (24 hrs) in Guidotti et al. (2000), their results may have been confounded by additional degradation of GAD67 protein in the schizophrenia group relative to the comparison group, accounting for the much larger observed reduction in GAD67 protein. Straub et al. (2007) reported no change in the DLPFC GAD67 protein levels. However, their results may also have been confounded by PMI, as long PMIs were present in both the comparison and schizophrenia groups (mean of 31 hrs and 36 hrs, respectively), which may have resulted in both groups falling to a floor level. In the current study, PMI is unlikely to have confounded the results since the mean PMI of the schizophrenia and comparison groups were nearly identical and all subjects had a PMI of less than 20 hours, a value at which GAD67 protein levels are well-preserved **(2.3.2)**.

Another potential reason that the results of the current study are discrepant with the literature is that differences in Western blotting methodology between this study and the two that have reported unchanged GAD67 protein levels (Straub et al., 2007; Dracheva et al., 2004) may have precluded the latter studies from detecting a difference. For example, Dracheva et al. used a pan GAD antibody, which produces bands at both 67 and 65 kDa, corresponding to GAD67 and GAD65, respectively. In my experience with this antibody, the small 2 kDa separation of these bands makes it difficult to prevent contamination between the two bands during quantification. Additionally, Straub et al. utilized actin as a normalizing protein, which I have shown is associated with twice the variability of tubulin **(2.3.4)**, the normalizer utilized here. In addition, the current study utilized a paired design with multiple replicates to further reduce variability, thus making it easier to detect a small difference. Finally, the current study utilized the LI-COR Odyssey Infrared Imaging System to visualize and quantify bands, an approach that has greater sensitivity and lower variability compared to the enzyme-based chemiluminescence utilized by both the Dracheva and Straub studies. In fact, a characterization of the LI-COR system at the antibody concentrations used in our study revealed a high degree of linearity as well as proportionality. In summary, our ability to detect to small differences in GAD67 protein that other studies failed to find highlights the importance of using a robust quantitative Western blotting strategy **(2.3)**.

### **5.3 WHAT ARE THE IMPLICATIONS OF LOWER GAD67 PROTEIN LEVELS AT PV AXON TERMINALS?**

#### **5.3.1 GAD67 protein is lower in PV<sub>b</sub> terminals; level in PV<sub>ch</sub> terminals is unclear**

The seminal dual ISH study demonstrating that approximately 50% of PV neurons lack detectable levels of GAD67 mRNA (Hashimoto et al., 2003) was not able to determine which subpopulation(s) of PV neurons are affected. That is, is lower GAD67 mRNA present in PV<sub>b</sub> cells, PV<sub>ch</sub> cells, or both? Below I will discuss the current evidence in favor of each of these outcomes.

Given that a subpopulation of PV interneurons does not contain detectable levels of GAD67 mRNA (Hashimoto et al., 2003), and GAD67 is important in the development of the proper number of PV axon terminals (Chattopadhyaya et al., 2007), a reduced density of PV-IR puncta, presumably PV<sub>b</sub> axon terminals (Erickson and Lewis, 2002), in layers 3-4 of subjects with schizophrenia (Lewis et al., 2001) suggests that PV<sub>b</sub> cells may contain lower GAD67 in schizophrenia. Until now, however, direct evidence that PV<sub>b</sub> cells contain less GAD67 has been lacking. In the current measurement of GAD67 protein in PV axon terminals (**3.4.4**), available evidence suggests that GAD67 was quantified only in PV<sub>b</sub> terminals. For example, a recent study in monkey DLPFC from our group using the same approach demonstrated that PV<sub>ch</sub> cartridges express mainly GAD67, and express very low levels of GAD65 (Fish et al., 2011). Although not yet quantified, the same scenario appears to be present in human tissue (Ken Fish, personal communication), suggesting that because we utilized GAD65 to mask and identify axon terminals, only PV terminals not associated with cartridges (presumably PV<sub>b</sub> terminals) were quantified. Thus, our data support the idea that GAD67 protein is lower in PV<sub>b</sub> terminals in schizophrenia.

The 49% reduction in GAD67 protein in PV<sub>b</sub> terminals is nearly 10X greater than the 5% reduction observed at the total protein level in the same subject pairs. Interestingly, all 5 pairs exhibit lower GAD67 protein at PV puncta in the schizophrenia subject, although only 2 of the pairs exhibited lower levels of total GAD67 protein. Although the sample size is small, these data suggest that some of the variability in the

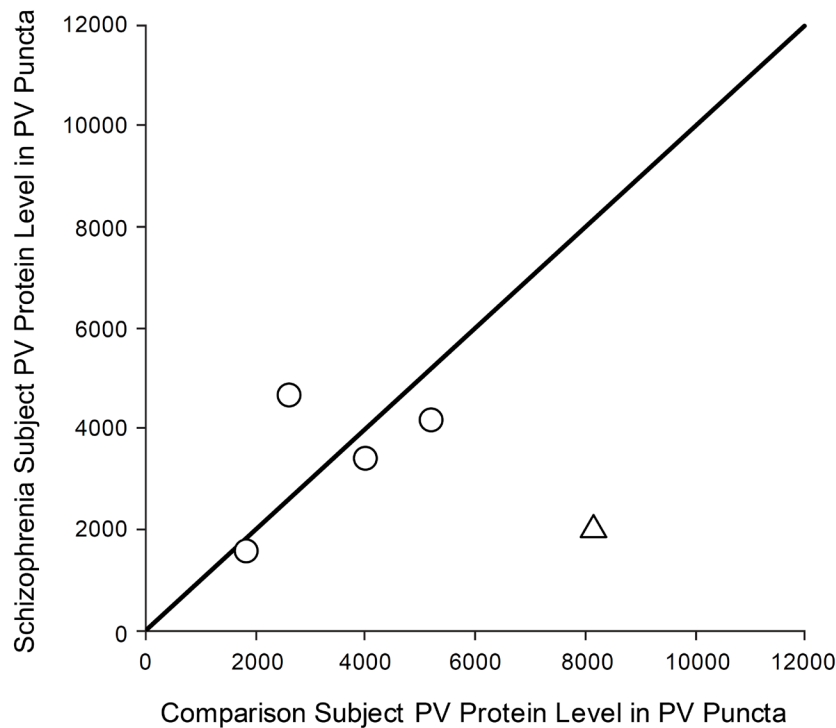
tissue homogenate measures may be eliminated by examining GAD67 in the specific subpopulation of neurons that are affected. Importantly, these data provide not only the first evidence of a GAD67 protein alteration in PV neurons in schizophrenia, but also the first evidence of a GAD67 decrement at the axon terminal, the site of GABA production and function. Moreover, these data mark the first time this powerful approach has been used in postmortem human tissue. Immunofluorescence and confocal microscopy, coupled with a novel methodology, have provided a robust assessment of GAD67 protein levels in a specific population of interneurons due to the use of a sensitive microscopy system, stereological sampling, an iterative threshold/morphological segmentation and data analysis approach that allows for the accurate quantification of a wide range of fluorescent intensities, and the elimination of the confounding autofluorescence of lipofuscin **(2.4)**. Going forward, this methodology will provide answers to a number of important questions that remain, some of which are described below.

Alterations in a variety of effectors of GABA neurotransmission have been interpreted as compensatory responses to lower GAD67 in PV<sub>ch</sub> cells (Lewis et al., 2005). First, correlated reductions in GAD67, PV, and GAT1 mRNA (Hashimoto et al., 2003; Volk et al., 2001) have been interpreted to mean that these changes are occurring in the same neurons. Additionally, the density of PV<sub>ch</sub> cartridges that are GAT1-IR is 40% lower in schizophrenia, although the density of other GAT1-IR structures is unaltered (Woo et al., 1998). Given that the changes in GAD67 and GAT1 seem to be present in the same neurons (Volk et al., 2001), this suggests that PV<sub>ch</sub> cells exhibit lower GAD67. This hypothesis has been further supported by the observation of increased GABA<sub>A</sub>  $\alpha$ 2-IR AIS number in schizophrenia (Volk et al., 2002), interpreted as a compensation to increase effects of deficient GABA synthesis at PV<sub>ch</sub> synapses. However, the hypothesis that PV<sub>ch</sub> cells contain lower GAD67 has never been directly demonstrated. Thus, it is currently unknown whether or not PV<sub>ch</sub> cells contain less GAD67, and future studies are needed to address this issue.



### 5.3.2 PV protein levels at PV<sub>b</sub> axon terminals

PV protein levels at PV<sub>b</sub> axon terminals were also measured in this study, by quantifying the PV fluorescence intensity at puncta triple-labeled for GAD67, PV, and GAD65. PV axon terminal protein levels were nonsignificantly 27.3% lower ( $t_4=0.894$ , one-tailed  $p=0.211$ ) in the DLPFC of subjects with schizophrenia (**Figure 23**). However, a decrease in PV protein levels should have been more detectable than a decrement in GAD67. In the same cohort of subjects in which we found a 15% reduction in GAD67 mRNA (**3.4.1**), the reduction in PV mRNA expression is larger in magnitude (22%; David Volk, personal communication), which is consistent with another report by our group (Hashimoto et al., 2008b). However, as shown in **Figure 23**, PV terminal levels exhibited substantially more variability than GAD67 axon terminal protein levels. In fact, the comparison subject of one pair seems to be driving the majority of the decrease, suggesting that PV levels may not be decreased in PV<sub>b</sub> cells. The increased variability in PV is consistent with the qPCR data in the current cohort (D. Volk, personal communication), as well as in a previous study (Hashimoto et al., 2008b). Thus, future studies with larger sample sizes are needed to accurately quantify PV protein levels in PV axon terminals.



**Figure 23. PV protein levels in PV puncta plotted for each pair of comparison and schizophrenia (circle) or schizoaffective (triangle) subjects.** Values below the diagonal unity line indicate lower PV protein in PV puncta in the schizophrenia subject relative to the comparison subject.

## 5.4 WHAT ETIOPATHOGENIC MECHANISMS UNDERLIE LOWER GAD67 IN SCHIZOPHRENIA, AND WHAT IS THE TIMING OF THE DEFICIT?

### 5.4.1 Potential mechanisms underlying lower GAD67

A thorough interpretation of the GAD67 deficit in schizophrenia requires knowledge of the underlying mechanism(s). Despite the widely-characterized GAD67 mRNA deficit, the etiology of this change is poorly understood. Although there are many possibilities, below I will discuss a few of the most compelling candidates.

#### **5.4.1.1 Allelic variation in GAD1**

The gene that encodes GAD67, GAD1, contains 18 exons across 45 kb of genomic DNA (Bu and Tobin, 1994) and is located on chromosome 2q31 (Bu et al., 1992). Recently, allelic variation in GAD1 has been implicated as a risk factor in schizophrenia. Several studies have associated single nucleotide polymorphisms (**SNPs**) in GAD1 with adult- (Straub et al., 2007; Du et al., 2008; Zhao et al., 2007) and childhood-onset schizophrenia (Addington et al., 2005), although others have reported no association (Lundorf et al., 2005; De Luca et al., 2004; Zhang et al., 2005). Additionally, GAD1 SNPs have been associated with deficits in attention and working memory, and with DLPFC activation (Straub et al., 2007). GAD1 SNPs have also been associated with GAD67 mRNA levels (Straub et al., 2007), indicating that alterations in the gene may underlie lower GAD67 mRNA in schizophrenia. Existing data suggest several possible mechanisms by which alterations in the GAD1 gene in schizophrenia may lead to lower transcription of GAD67. Although no risk allele SNPs are located within the coding region of GAD1 (Akbarian and Huang, 2006), a risk SNP located in the promoter region is associated with increased transcription factor binding, which may lead to altered promoter function (Zhao et al., 2007). Additionally, epigenetic mechanisms, which control gene activity without altering the DNA sequence, may also play a role. Methylation of certain lysine residues on nuclear histone proteins, around which DNA is wound, result in shifts between active and repressed transcription (reviewed in Tsankova et al., 2007). In female schizophrenia subjects, GAD1 risk SNPs are associated with lower PFC GAD67 mRNA and a shift from active to repressed histone methylation (Huang et al., 2007), providing a potential mechanism through which allelic variation may lower GAD67 mRNA levels. However, the gender-specificity of the finding suggests that this mechanism may only be occurring in females. Therefore, the exact role of epigenetic mechanisms in GAD1 transcription remains to be elucidated.

Although there is evidence implicating allelic variation in the GAD1 gene as a potential pathogenic mechanism, low penetrance, small effect sizes and a lack of concordance between studies regarding the specific SNPs involved suggest that allelic variation in GAD1 may play a contributing role in only a minority of cases and is not the only causative factor underlying lower GAD67 in schizophrenia (Insel, 2010). Further

confusing the issue, schizophrenia GAD1 risk alleles have recently been associated with *increased* GABA levels in the anterior cingulate cortex of healthy subjects (Marenco et al., 2010). Thus, the exact role of GAD1 risk alleles in the pathogenesis of GAD67 deficits in schizophrenia remains to be determined. While allelic variation in GAD1 may play a role in lower GAD67 in schizophrenia, other mechanisms must also be at work.

#### **5.4.1.2 Reduced signaling through TrkB**

The allelic variations in GAD1 described above support the idea that a GAD67 deficit is a primary, causative factor in the illness. Alternatively, lower GAD67 may be the consequence of a more upstream mechanism. One such mechanism that may be responsible for reductions in GAD67, as well as in PV and GAT1, in schizophrenia is reduced signaling through the TrkB receptor and its ligand brain-derived neurotrophic factor (**BDNF**). TrkB/BDNF signaling is important for the development of interneurons (Woo and Lu, 2006), especially PV cells, which comprise the majority of TrkB-expressing interneurons (Gorba and Wahle, 1999; Cellerino et al., 1996). For example, TrkB/BDNF signaling has been implicated in a wide variety of developmental processes, including interneuron migration, differentiation, and synaptogenesis (Woo and Lu, 2006; Huang et al., 1999; Kohara et al., 2003; Kohara et al., 2007). BDNF treatment *in vitro* upregulates GAD and promotes GABA release (Yamada et al., 2002), and BDNF overexpressing mice exhibit accelerated PV interneuron maturation (Huang et al., 1999).

Both BDNF and TrkB mRNA are lower in the DLPFC of subjects with schizophrenia (Hashimoto et al., 2005; Weickert et al., 2003; Weickert et al., 2005). In fact, in the same subject pairs, the within-pair changes in TrkB mRNA are significantly correlated with those of both GAD67 and PV (Hashimoto et al., 2005). Additionally, TrkB hypomorphic, but not BDNF KO, mice exhibit lower levels of GAD67 and PV mRNA (Hashimoto et al., 2005), suggesting that reduced signaling through the TrkB receptor may underlie lower levels of both GAD67 and PV in schizophrenia. Furthermore, TrkB hypomorphs exhibit a reduced density of GAD67 mRNA+ cells, without a change in the mRNA levels per cell (Hashimoto et al., 2005), similar to the pattern observed in

schizophrenia (Volk et al., 2000). Although GAT1 has not been examined in these animals, it also appears to be modulated by the TrkB/BDNF signaling pathway. Inhibition of tyrosine kinases in hippocampal cells reduces GAT1 phosphorylation and GABA reuptake, while application of BDNF upregulates GAT1 function (Law et al., 2000).

#### **5.4.1.3 NMDA hypofunction in PV neurons**

Glutamate-mediated excitatory neurotransmission occurs through a variety of fast ionotropic (NMDA, AMPA, and kainate) receptors, as well as the slower G protein-coupled metabotropic receptors. The now widely-recognized NMDA receptor hypofunction hypothesis of schizophrenia originated from the observation that NMDA receptor antagonists mimic schizophrenia symptoms, and has been strengthened by findings of lower levels of glutamate receptors, such as the NR1 subunit, in schizophrenia, as well as studies demonstrating that several schizophrenia risk genes, such as neuregulin 1 and ErbB4, affect NMDA receptor signaling (reviewed in Lewis and Moghaddam, 2006).

Interneurons are known to depend on NMDA-mediated excitation (Jones and Bühl, 1993; Maccaferri and Dingledine, 2002), and thus lower glutamatergic drive onto GABA neurons may result in subsequent reductions in markers of inhibitory transmission (Lewis and Moghaddam, 2006). The vast majority (80-90%) of PV neurons express NR1 receptors (González-Albo et al., 2001; Huntley et al., 1994), and thus PV neurons are thought to be particularly affected by reduced NMDA receptor signaling (Lewis and Moghaddam, 2006). In fact, NMDA receptor antagonists reduce GAD67 (Behrens et al., 2007), PV (Cochran et al., 2003; Behrens et al., 2007), and GAT1 (Bullock et al., 2009) mRNA. Additionally, mice with a selective knockout of the NR1 receptor on ~50% of cortical and hippocampal interneurons, presumably mostly PV neurons, during early postnatal development exhibit reduced GAD67 and PV protein (Belforte et al., 2010). Moreover, mice with a selective knockout of NR1 on PV neurons exhibit alterations in  $\gamma$  oscillations (Carlén et al., 2011), which are disturbed in schizophrenia (Cho et al., 2006).

However, there are limitations of this version of the NMDA hypofunction hypothesis. First, the hypothesis is based largely on the observation that treatment with NMDA receptor antagonists in adults mimics the symptoms of schizophrenia in healthy subjects, and worsens the symptoms of schizophrenia subjects. However, GAD67 and PV protein levels are not affected when NR1 is knocked out during adulthood (Belforte et al., 2010), consistent with the finding that in adult mice NMDA receptors make only a weak contribution to PV cell excitatory postsynaptic currents (**EPSCs**) (Rotaru et al., 2011; but see Carlén et al., 2011). A second limitation of the NMDA hypofunction hypothesis is that it predicts that reduced signaling through NR1 receptors on PV neurons leads to disinhibition of pyramidal neurons (Lisman et al., 2008). In fact, substantial evidence points not to an increase, but rather a *decrease*, in activity in the DLPFC. The majority of imaging studies suggest hypofunction of this brain region (Weinberger, 2001), and several studies have documented a lower power of  $\gamma$  oscillations as well (Cho et al., 2006; Minzenberg et al., 2010).

#### **5.4.1.4 Reduced excitatory neurotransmission**

In addition to the alterations in inhibitory interneurons that are present in the DLPFC of subjects with schizophrenia, a number of changes are also present in excitatory pyramidal cells. First, there is a significant reduction in the density of dendritic spines, a finding that is most pronounced in layer 3 (Glantz and Lewis, 2000; Kolluri et al., 2005). Additionally, although the total number of pyramidal neurons is not altered in the PFC (Akbarian et al., 1995b; Thune et al., 2001), somal size is significantly lower (Glantz and Lewis, 2000; Pierri et al., 2001; Rajkowska et al., 1998), and neuropil (Selemon and Goldman-Rakic, 1999) and dendritic tree size (Black et al., 2004; Kalus et al., 2000) are reduced. In addition to these morphological alterations in pyramidal neurons, a number of molecular changes have also been found. The mRNA levels for two spine markers, Duo and cell division cycle 42 (**Cdc42**), are lower in the DLPFC of subjects with schizophrenia, and are significantly correlated with the reduction in layer 3 spine density (Hill et al., 2006). In addition, immunoreactivity of synaptophysin, a synaptic vesicle protein, is also reduced in the DLPFC (Glantz and Lewis, 1997), suggestive of a disturbance in synaptic transmission.

The pyramidal cell alterations described above point to a reduction in excitatory function in the DLPFC of subjects with schizophrenia. Given that changes in both excitatory and inhibitory neurotransmission are found in schizophrenia, one hypothesis currently gaining momentum is that the reduced excitatory activity is an “upstream” pathology that produces compensatory downregulations in inhibitory activity, in an attempt to maintain the appropriate balance of excitatory and inhibitory activity (**E/I balance**) needed for proper cortical functioning (Lewis et al. in press). E/I balance is important to prevent both runaway excitatory activity (too much excitation) and a dying out of cortical activity (too much inhibition) (Turrigiano and Nelson, 2004). This balance is maintained through the scaling of excitatory and inhibitory strength, in a process termed homeostatic synaptic plasticity (Pozo and Goda, 2010). Scaling occurs through diverse mechanisms such as alterations in neurotransmitter content and postsynaptic receptor number (Pozo and Goda, 2010; Kilman et al., 2002). For example, activity deprivation using tetrodotoxin has been shown to reduce the amplitude of mIPSCs (Kilman et al., 2002). Thus, reduced excitatory activity of pyramidal cells may result in compensatory downregulation of inhibitory activity onto these neurons, as evidenced by lower GAD67 levels in PV cells.

In fact, GAD7 is activity-dependent (Benson et al., 1994), and reductions in excitatory signaling have been shown to produce many of the same alterations in inhibitory function observed in the illness. For example, rats with neonatal lesions to the ventral hippocampus, which sends excitatory inputs to the PFC, exhibit lower GAD67 mRNA levels (Lipska et al., 2003). Additionally, using tetrodotoxin, monocular deprivation of the lateral geniculate nucleus of the thalamus as well as the visual cortex results in lower GAD67 mRNA in primates (Huntsman et al., 1995). Interestingly, NR1 KO mice exhibit a reduced spine density in layer 2/3 pyramidal neurons (Ultanir et al., 2007). In addition to their localization on PV neurons, NR1 receptors are also located on the soma and dendrites of pyramidal neurons (Huntley et al., 1994), suggesting that perhaps NMDA hypofunction in schizophrenia is occurring on pyramidal neurons rather than on PV neurons. Thus, the aforementioned downregulations in PV neurons (lower GAD67 and PV) after treatment with NMDA antagonists may be occurring as a response to reduced excitation of pyramidal neurons.

#### 5.4.2 Timing of the GAD67 deficit

A critical issue in interpreting lower GAD67 in schizophrenia is when the deficit in GAD67, and presumably GABA, arises. Since GAD67 plays a role in development, an earlier vs. later reduction in GAD67 would have differing functional consequences. Early knockdown of GAD67 in PV<sub>b</sub> cells, during the development of PV synaptic architecture, as well as a germline reduction in GAD67 results in reduced axonal branching and arborization, and reduced bouton density (Chattopadhyaya et al., 2007). However, performing the same manipulation during adulthood does not produce the same synaptic deficits (Chattopadhyaya et al., 2007). Therefore, a GAD67 deficit in schizophrenia arising early in development would be expected to produce fewer numbers of PV<sub>b</sub> terminals. Both the reduced density of PV-IR puncta (Lewis et al., 2001) and lower levels of  $\alpha 1$  mRNA (Glausier and Lewis, 2011), which may reflect lower numbers of  $\alpha 1$ -containing GABA<sub>A</sub> receptors and thus fewer inputs from PV<sub>b</sub> cells, in schizophrenia suggests that the number of PV terminals may be reduced. Alternatively, these data may reflect lower expression per terminal, resulting in some that fall below detectability, rather than a loss of terminals. Furthermore, since PV<sub>b</sub> axons contact not only pyramidal neurons but also other PV<sub>b</sub> cells (Melchitzky et al., 1999), a lower number of PV terminals resulting from lower GAD67 early in development would be expected to reduce  $\alpha 1$  mRNA on both targets, since the detrimental effects of lower GAD67 should be present in both locations. However,  $\alpha 1$  mRNA levels were significantly reduced in pyramidal neurons but not interneurons (Glausier and Lewis, 2011).

In the current study of GAD67 protein levels in PV axon terminals (**3.3.4**), we did not observe a significant difference in the density of GAD67+/PV+/GAD65+ puncta between comparison (4.0 per 1000  $\mu\text{m}^3$ ) and schizophrenia (4.4 per 1000  $\mu\text{m}^3$ ) subjects ( $t_4 = -0.526$ ,  $p = 0.627$ ). Since we could not estimate any tissue volume changes in schizophrenia, and therefore density measures are potentially confounded, there are caveats with these data, and thus their interpretative value may be limited. However, if they are real, it suggests that a GAD67 deficit in schizophrenia is occurring later in development, after GAD67 has contributed to bouton development.



The deficit in GAD67 does not appear to be progressive across the lifespan of the individual for a number of reasons. First, there was no significant correlation between GAD67 mRNA and age ATOD ( $r=-0.25$ ,  $p=0.104$ ; **3.4.1**) Although others have reported a similar, negative correlation between GAD67 mRNA levels and age that did reach statistical significance (Straub et al., 2007), we also did not observe a significant difference in GAD67 mRNA between the youngest and old quarter of schizophrenia subjects, compared using either a t test or an ANCOVA ( $t_{18}=1.586$ ,  $p=0.130$ ;  $F_{1,14}=1.465$ ,  $p=0.246$ ). Additionally, there was no significant correlation between GAD67 mRNA and age-corrected length of illness (**3.4.1**). Duncan et al. (2010) reported a significant negative correlation between GAD67 mRNA and length of illness, though this may be confounded by their reported significant negative effect of age. In summary, our data suggest that the GAD67 mRNA deficit is stable not only throughout the lifespan of an individual, but also throughout the course of the illness. Importantly, this suggests that lower GAD67 in schizophrenia is not a consequence of having the illness, and supports the idea that lower GAD67 underlies impairments in  $\gamma$  oscillations and cognition. Notably,  $\gamma$  oscillation impairments and cognitive deficits are also present in both first episode (Minzenberg et al., 2010) and chronic (Cho et al., 2006) schizophrenia patients.

## **5.5 WHAT ARE THE CONSEQUENCES OF LOWER GAD67 IN PV<sub>B</sub> CELLS?**

### **5.5.1 Lower PV and GAT1 mRNA are not a consequence of lower GAD67 in PV neurons**

Since PV and GAT1 can each reduce the level of GABA available to postsynaptic receptors, it has been hypothesized that their reduction in schizophrenia may be a compensation to counteract upstream reductions in GAD67 (Lewis et al., 2005). Based on the results obtained in **Chapter 4**, lower PV and GAT1 mRNA do not seem to be a consequence of lower GAD67 in PV neurons, although we cannot rule out the possibility that differences between mouse and humans may have prevented detection of an

association **(4.5)**. As reviewed above **(5.4)**, it is possible that an upstream mechanism such as reduced signaling through TrkB receptors or NMDA hypofunction on PV neurons may result in concomitant changes in GAD67, PV, and GAT1. Another third possibility is that PV and GAT1 are lower specifically in PV<sub>ch</sub> cells (Lewis et al., 2011), which recent evidence suggests may play an excitatory role in the cortex.

Due to the exclusive input of PV<sub>ch</sub> cells onto the AIS, the site of axon potential generation, of pyramidal neurons, PV<sub>ch</sub> cells have classically been thought to play a powerful role in the inhibition of pyramidal cells (Miles et al., 1996). However, in the last 5 years, evidence pointing to a depolarizing role of PV<sub>ch</sub> cells has accumulated (reviewed in Woodruff et al., 2010). In 2006, the first report of a depolarizing effect of PV<sub>ch</sub> cells was published, in which the authors reported that stimulation of PV<sub>ch</sub> cells results in an excitation of postsynaptic pyramidal neurons (Szabadics et al., 2006). In addition, it was shown that the reversal potential (**E<sub>GABA</sub>**) in these cells was greater than the resting membrane potential (**V<sub>rest</sub>**), likely due to a lack of the chloride extruding potassium/chloride cotransporter 2 (**KCC2**) at the AIS (Szabadics et al., 2006). A second study showed that pyramidal cells contain an E<sub>GABA</sub> gradient, with lowest E<sub>GABA</sub> levels (producing hyperpolarization) at dendrites, and highest E<sub>GABA</sub> levels (producing depolarization) at the axon (Khirug et al., 2008). However, by measuring extracellular field potentials, a less invasive method than the previous two studies, a purely inhibitory function of PV<sub>ch</sub> cells has recently been demonstrated (Glickfeld et al., 2009). Importantly, neither of the two studies that demonstrated an excitatory function of PV<sub>ch</sub> cells ruled out an inhibitory function. Thus, the role of PV<sub>ch</sub> neurons in cortical function is still unknown, and future studies are needed to definitively determine whether they are excitatory, inhibitory, or both (Woodruff et al., 2010).

In light of the recent evidence that PV<sub>ch</sub> cells may depolarize their pyramidal cell targets, it is possible the PV and GAT1 are lower in these cells in an attempt to counteract upstream reductions in pyramidal cell excitation and restore E/I balance in schizophrenia (Lewis et al., 2011). Lower PV and GAT1 in excitatory PV<sub>ch</sub> cells would function to increase excitation of pyramidal cells, although ultimately this compensation is insufficient to restore pyramidal cell activity, since  $\gamma$  oscillations and cognitive control deficits are still present in schizophrenia (Lewis et al., 2005).

This E/I balance hypothesis would predict that PV and GAT1 levels would be normal in PV<sub>b</sub> cells. The current nonsignificant change in PV protein levels in PV puncta **(5.4.2)**, though potentially confounded by small sample size, suggests that this may be the case. However, one interpretation of the reduced density of PV-IR puncta, presumably PV<sub>b</sub> axon terminals (Erickson and Lewis, 2002), in layers 3-4 of subjects with schizophrenia (Lewis et al., 2001) is that lower PV levels in these cells fall below the threshold of detectability. In fact, given that PV expression is activity dependent in the visual cortex (Blümcke et al., 1994; Pinheiro Botelho et al., 2006), it is possible that PV is downregulated in PV<sub>b</sub> cells in response to reduced activity in these cells resulting from lower GAD67. However, there are two problems with this interpretation. First, lower PV in PV<sub>b</sub> cells would have the net effect of increasing GABA neurotransmission in these cells, counteracting the reduction of GABA neurotransmission in PV<sub>b</sub> cells in response to reduced excitation in pyramidal cells. Second, this interpretation is inconsistent with the lack of change in PV and GAT1 mRNA that we obtained in the mouse models of lower GAD67 **(Chapter 4)**. It is possible that a nonselective reduction in GAD67 in both PV<sub>b</sub> and PV<sub>ch</sub> cells in the mouse models precluded the detection of a difference in PV levels.

Alternatively, the lower density of PV-IR puncta in layers 3 and 4 in schizophrenia may not reflect lower PV levels, and instead may reflect fewer inputs from the medial dorsal **(MD)** nucleus of the thalamus, (Lewis et al., 2001). PV is expressed in excitatory neurons of the thalamus that project to layers 3 and 4 of the PFC (Jones, 1998; Giguere and Goldman-Rakic, 1988). Several studies have reported reduced volume and neuronal number in the MD thalamus (Pakkenberg, 1990; Young et al., 2000), however more recent studies are mixed (reviewed in Byne et al., 2009). Furthermore, in rats, lesions of the MD nucleus do not produce alterations in PFC GAD67 mRNA (Volk and Lewis, 2003). Thus, the status and consequences of MD thalamic alterations in schizophrenia is currently unclear, and therefore whether the lower density of PV-IR puncta reflects deficits in thalamic or PV<sub>b</sub> terminals is unknown.

In contrast to the conflicting data for PV, the lower density of GAT1-IR cartridges (Pierri et al., 1999), but lack of change in GAT1-IR puncta (presumably all other non-

cartridge puncta, including those from PV<sub>b</sub> cells) in schizophrenia (Woo et al., 1998), suggests that GAT1 may be selectively reduced in PV<sub>ch</sub> but not PV<sub>b</sub> cells.

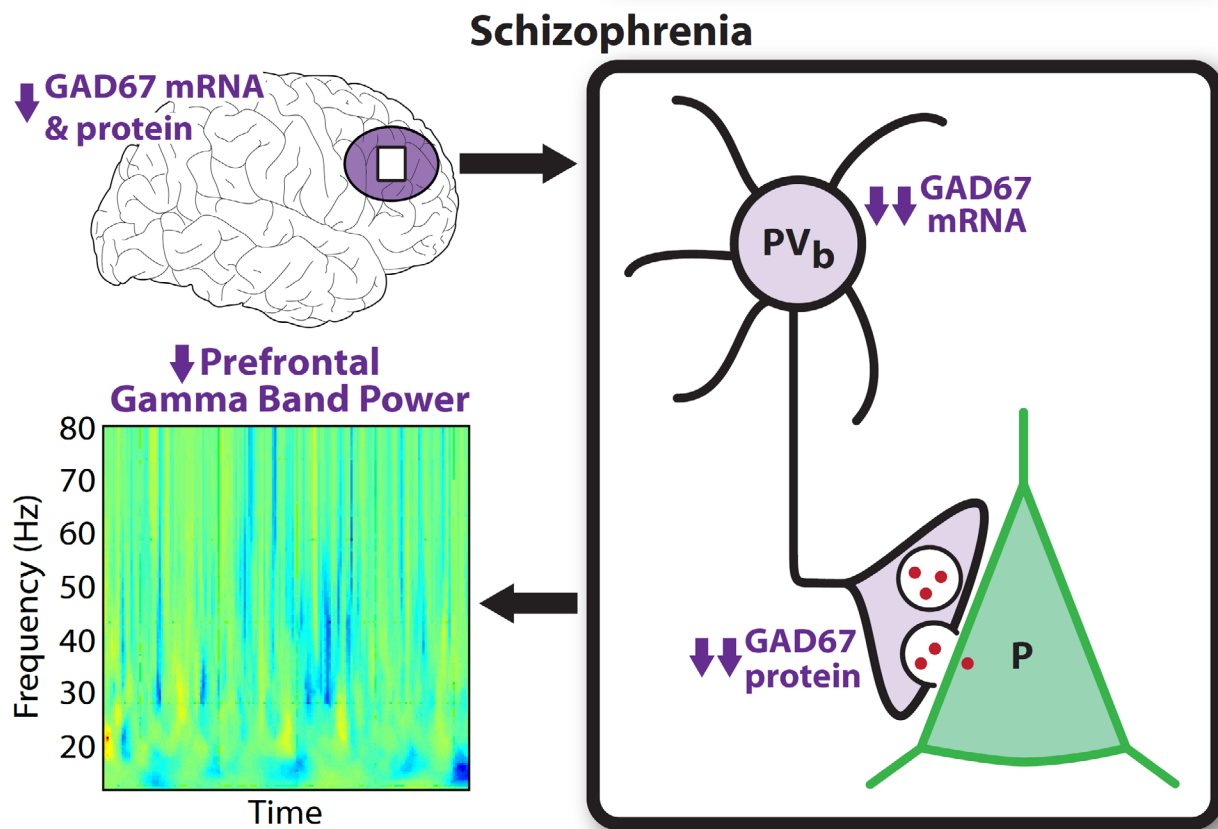
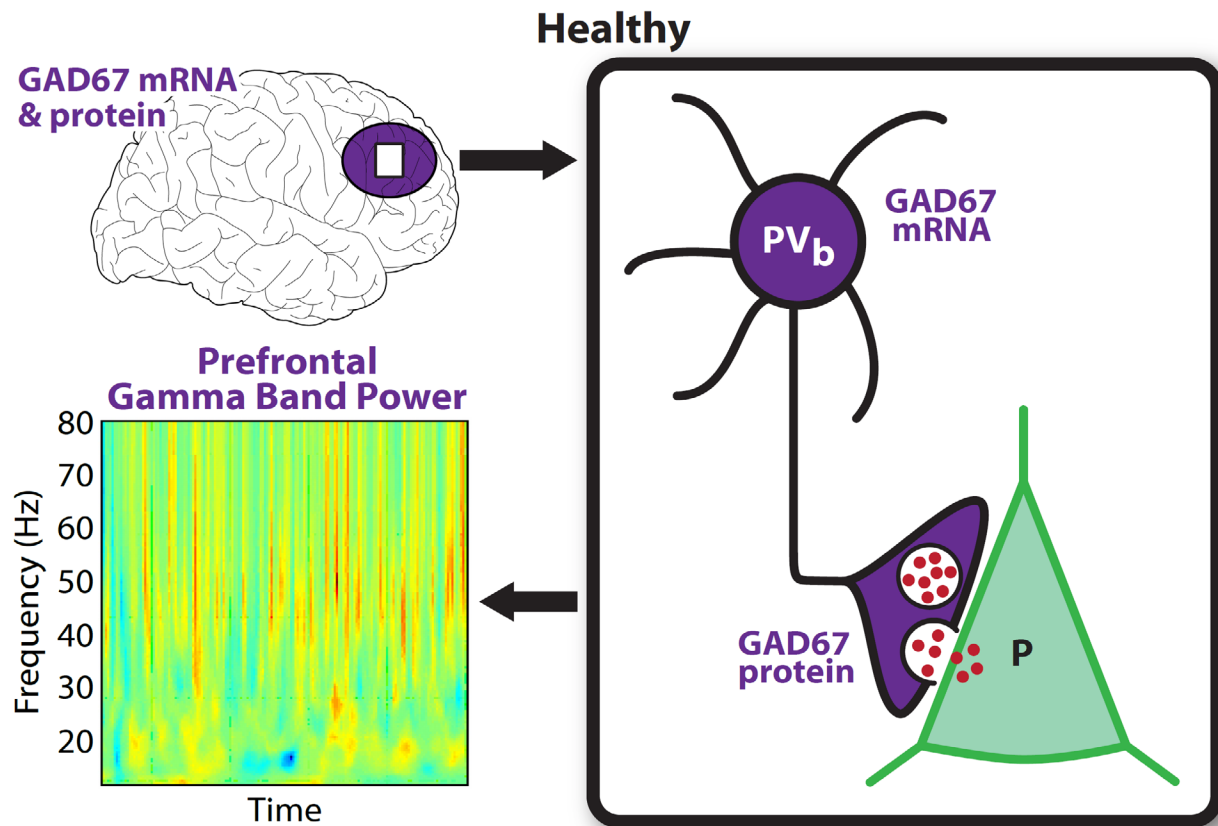
Importantly, this E/I balance hypothesis provides a potential explanation as to why lower PV and GAT1 mRNA were not observed following reductions in GAD67 (**Chapter 4**). Lower PV and GAT1 mRNA in PV<sub>ch</sub> cells may be a response to reduced excitatory drive in pyramidal neurons, not a consequence of lower GAD67. Studies that can definitively establish PV and GAT1 protein levels in PV<sub>b</sub> and PV<sub>ch</sub> axon terminals separately are an important next step in advancing this hypothesis. PV and GAT1 expression levels would be expected to be unchanged in PV<sub>b</sub> cells, but decreased in PV<sub>ch</sub> cells. Using similar methodologies as what we employed in **3.4.4**, it is possible to selectively measure protein levels at synapses furnished by the two different cell types. This can be accomplished using antibodies against proteins such as GAT1 that selectively label cartridges, which are visually distinguishable from the PV<sub>b</sub> axon terminals that appear as puncta (Fish et al., 2011).

### **5.5.2 Lower GAD67 in PV<sub>b</sub> cells underlies impaired $\gamma$ oscillations and cognitive control deficits in schizophrenia**

PV neurons are known to be crucial in the generation of  $\gamma$  oscillations, the synchronized activity of networks of pyramidal neurons at 30-80 Hz, thought to underlie cognitive control (reviewed in Gonzalez-Burgos, 2010). For example, the  $\gamma$  local field potential is tightly coupled to the firing of PV neurons both *in vitro* (Hájos et al., 2004) and *in vivo* (Tukker et al., 2007). Moreover, recent evidence using optogenetic techniques has demonstrated that suppression and activation of PV neurons suppresses and generates, respectively,  $\gamma$  activity *in vivo*, indicating that PV neurons are both necessary and sufficient for the generation of  $\gamma$  oscillations (Sohal et al., 2009; Cardin et al., 2009). In fact, fast spiking PV<sub>b</sub> cells are known to be especially critical for  $\gamma$  oscillations (Gonzalez-Burgos, 2010). The GABA<sub>A</sub>  $\alpha$ 1-containing receptors that predominate at PV<sub>b</sub> cell synapses exhibit decay kinetics that are consistent with the firing rate of  $\gamma$  oscillations (Gonzalez-Burgos and Lewis, 2008). Furthermore, the firing of PV<sub>b</sub> cells is

more strongly coupled to the  $\gamma$  oscillation cycle than the firing of either PV<sub>ch</sub> or CCK cells (Gulyas et al. 2010).

Importantly,  $\gamma$  oscillations are thought to underlie higher order cognitive processes (Fries, 2009). For example,  $\gamma$  band activity is induced during the delay period of working memory tasks (Tallon-Baudry et al., 1998), and the power of  $\gamma$  synchrony increases in proportion to working memory load (Howard et al., 2003). Thus, given the crucial role of PV<sub>b</sub> neurons in the generation of  $\gamma$  oscillations, the alterations in PV<sub>b</sub> neurons demonstrated here are a plausible mechanism underlying the lower  $\gamma$  band power, and cognitive control deficits, observed in the illness (**Figure 24**).



**Figure 24. Schematic summary, and predicted functional consequences, of lower GAD67 mRNA and protein in the DLPFC of subjects with schizophrenia.** In healthy subjects, normal tissue levels of GAD67 mRNA and protein in DLPFC area 9 (purple shaded area, top left) are associated with normal levels of GAD67 mRNA and protein in PV-containing cell bodies and axon terminals, respectively, and normal levels of GABA release from synaptic vesicles onto pyramidal (P) neurons (top right), providing the inhibitory inputs required for normal  $\gamma$  band power as measured by electroencephalography during cognitive control tasks (top left). In schizophrenia, modest reductions in tissue levels of GAD67 mRNA and protein (bottom left) are associated with pronounced reductions of GAD67 mRNA and protein in PV neurons and axon terminals, respectively (bottom right). The predicted reduction in GABA (red dots) available to be released from synaptic vesicles (bottom right) leads to the lower  $\gamma$  band power present over the DLPFC during cognitive control tasks (bottom left). Warmer colors in the heat maps of  $\gamma$  band power indicate higher power and cooler colors indicate lower power. Heat maps are from Cho et al. Copyright, 2006, Proceedings of the National Academy of Sciences.

## 5.6 CONCLUDING REMARKS

Lower GAD67 mRNA in schizophrenia has been replicated by numerous studies since the initial report was published in 1995 (Akbarian et al.). Nearly a decade later, it was demonstrated that lower GAD67 mRNA is present in PV neurons (Hashimoto et al., 2003). However, a number of important aspects of this deficit have remained undefined. Accordingly, this dissertation examined factors that may contribute the heterogeneity of the mRNA deficit, total protein levels of GAD67 as well as those in PV axon terminals, as well as the consequences of lower GAD67 in PV neurons. Following the development of innovative solutions to the challenges associated with measurement of protein in postmortem human tissue, we found the following four key results:

**1) Lower GAD67 mRNA is common in schizophrenia and is not associated with potential confounding factors such as medication use or substance abuse; predictors of illness severity or measures of functional outcome; or illness chronicity (3.4.1).** Using qPCR in the largest cohort to date, we did not find a relationship between GAD67 mRNA and these factors. Thus, the lack of a GAD67 mRNA deficit in roughly 25% of schizophrenia patients is likely to result from additional causes. Importantly, lower GAD67 does not seem to be a consequence of illness treatment or chronicity.

**2) Total GAD67 protein is significantly reduced in schizophrenia (3.4.2).** We utilized a Western blotting methodology that provided a robust quantification of total GAD67 protein levels due to the use of only subjects with PMIs in which GAD67 protein is well-preserved, a paired study design with multiple replicates, the use of a stable normalizer, and the linearity and proportionality of the antibodies used. We found that GAD67 protein levels are significantly lower in the DLPFC of subjects with schizophrenia, with a magnitude that is similar to the mRNA deficit.

**3) GAD67 protein levels in PV axon terminals, the site of GABA production and function, are substantially reduced in the DLPFC of subjects with schizophrenia (3.4.4).** We used immunofluorescence and confocal microscopy, coupled with a novel methodology, to provide a robust assessment of GAD67 protein levels in a specific population of interneurons due to the use of a sensitive microscopy system, stereological sampling, an iterative threshold/morphological segmentation and data analysis approach that allows for the accurate quantification of a wide range of fluorescent intensities, and the elimination of the confounding autofluorescence of lipofuscin. The reduction in GAD67 protein observed in PV axon terminals was much greater than that observed at the total protein level.

**4) Lower PV and GAT1 mRNA are not a consequence of lower GAD67 in PV neurons (4.4).** Using ISH in two different mouse models of lower GAD67, one with lower GAD67 specifically in PV neurons ( $PV^{GAD67+/-}$ ) and the other with lower GAD67 in



all interneurons ( $\text{GABA}^{\text{GAD67+/-}}$ ), we examined the consequences of lower GAD67. Neither PV nor GAT1 mRNA were altered at the tissue level in adult or developmental  $\text{PV}^{\text{GAD67+/-}}$  mice. Additionally, cellular analyses confirmed a lower level of GAD67, but not of PV, mRNA in these animals. Finally, PV and GAT1 mRNAs were also unaltered in  $\text{GABA}^{\text{GAD67+/-}}$  mice. Taken together, these data suggest that lower GAD67 levels do not result in changes in PV or GAT1, and therefore lower GAD67 is unlikely to be the cause of reduced PV and GAT1 mRNA in schizophrenia.

Together, these data provide an important characterization of the GAD67 deficit in schizophrenia. The findings that lower GAD67 mRNA expression is common in schizophrenia, is not a consequence of illness treatment or chronicity, and leads to less translation of the protein, especially in the axon terminals of  $\text{PV}_b$  neurons, support the hypothesis that lower GABA synthesis in  $\text{PV}_b$  neurons contributes to DLPFC dysfunction, impaired  $\gamma$  oscillations, and cognitive control deficits in schizophrenia. Additionally, the lack of change in PV and GAT1 mRNA in two different mouse models of lower GAD67 suggests that these transcripts are not reduced in schizophrenia as a consequence of lower GAD67.

## **APPENDIX A**

### **SUPPLEMENTARY METHODS**

#### **A.1 GAD67 MRNA LEVELS**

##### **Tissue collection and preparation**

The right hemisphere of each brain was blocked coronally, frozen immediately, and stored at -80°C (Volk et al., 2000). Cryostat sections from the anterior-posterior level corresponding to the middle portion of the superior frontal sulcus were cut serially and confirmed to contain DLPFC area 9 from adjacent Nissl-stained sections using cytoarchitectonic criteria (Volk et al., 2000). Cortical gray matter was dissected in a manner that excluded white matter contamination and provided excellent RNA preservation, and collected into tubes containing Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated from Trizol homogenates, cleaned by RNeasy columns (Qiagen, Valencia, CA), and used for qPCR, as described below. RIN and pH were determined as described previously (Hashimoto et al., 2008a).

##### **Quantitative PCR**

cDNA was synthesized from total RNA for each subject using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). All primer pairs (**Table**

**3)** demonstrated: 1) high amplification efficiency (>96%) across a wide range of cDNA dilutions; 2) specific single products in dissociation curve analysis; and 3) melting temperatures similar to those predicted by oligonucleotide software. Quantitative PCR was performed with Power SYBR Green dye and an ABI StepOne Plus Real Time PCR System (Applied Biosystems). The difference in cycle threshold (dCT) for GAD67 mRNA expression was calculated by subtracting the geometric mean of the cycle threshold for the three reference genes from the cycle threshold of GAD67 mRNA. Because this dCT represents the log2-transformed expression ratio of the target transcript to the geometric mean of the 3 reference genes, the relative expression level of GAD67 mRNA was determined as  $2^{-dCT}$  (Hashimoto et al., 2008b; Vandesompele et al., 2002).

**Table 3. Design of the 4 primer sets used in qPCR<sup>a</sup>**

Gene	Accession #	Amplicon Size (bp)	Position	Forward Primer (F) Reverse Primer (R)
BA	NM_001101	101	1146-1246	(F) GATGTGGATCAGCAAGCA (R) AGAAAGGGTGTAACGCAACTA
CyPA	NM_021130	126	159-284	(F) GCAGACAAGGTCCCAAAG (R) GAAGTCACCACCCTGACAAC
GAPDH	NM_002046	87	556-642	(F) TGCACCACCAACTGCTTAGC (R) GGCATGGACTGTGGTCATGAG
GAD67	NM_000817	86	2495-2580	(F) GTTTCCCGCTCCAAGAGAAT (R) TGGAGTTGTTGGACAAGCTG

<sup>a</sup>Abbreviations: bp, base pairs; BA, beta actin; CyPA, cyclophilin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAD67, glutamic acid decarboxylase 67 kDa

## **A.2 GAD67 PROTEIN LEVELS**

### **Tissue collection and preparation**

Gray matter from the right hemisphere of each brain was obtained as described above and collected into tubes for total protein analysis (average of 22 mg of tissue per tube). Tissue was homogenized by sonication in a volume of SDS buffer (125mM Tris pH 7, 2% SDS, 10% glycerol) equal to 10 times the tissue weight. Protein was extracted from homogenates by incubation at 70°C for 10 min with occasional vortexing, followed by centrifugation at 16,000 g for 10 min. This method allows for optimal protein extraction with minimal degradation (Ericsson et al., 2007). The supernatant was collected and an aliquot was used for total protein concentration determination in triplicate using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. The remaining supernatant from protein extraction was frozen in aliquots. A single aliquot was then used to prepare samples in 1X Protein Loading Buffer (LI-COR Biosciences, Lincoln, NE) containing 10%  $\beta$ -mercaptoethanol that were stored in single-use aliquots at -20°C. To limit its effect on protein levels, samples were exposed to only 2 freeze-thaw cycles.

### **Western blotting**

Unless otherwise noted, all reagents were obtained from Thermo Fisher Scientific, all incubation steps were performed at room temperature, and all washes were performed for 5 min each in Tris-buffered saline with Tween-20 (10mM Tris pH 7.5, 150mM NaCl, 0.05% Tween 20).

After denaturing at 70°C for 5 min, samples were resolved by SDS PAGE in 10% Precise Protein Gels in Tris-HEPES SDS running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS) at 60-62 volts for 2.5 hrs. Proteins were transferred to nitrocellulose membrane (LI-COR Biosciences) at 85 volts for 45 min in Tris-Glycine blotting buffer (25mM Tris, 192mM glycine, 20% methanol). Membranes were blocked for 1 hr in 50% Odyssey® Blocking Buffer (LI-COR Biosciences) and 50% Tris-buffered saline, and

washed once. Membranes were then incubated in a primary antibody mixture containing  $\beta$ -tubulin (mouse, 1:20,000, Millipore, Billerica, MA) and GAD67 (goat, 1:800, R&D Systems, Minneapolis, MN) in SuperBlock Blocking Buffer with 0.05% Tween 20 overnight at 4°C, and subsequently washed 5 times before incubation for 1 hr in a mixture of secondary antibodies containing IRDye® donkey anti mouse 680 and IRDye® donkey anti goat 800 (both 1:20,000; LI-COR Biosciences) in 50% Odyssey® Blocking Buffer and 50% Tris-buffered saline with Tween-20 and 0.01% SDS. Following 5 washes, signal was detected using the LI-COR Odyssey® Infrared Imaging System.

### **A.3 GAD67 PROTEIN LEVELS AT PV PUNCTA**

#### **Tissue collection and preparation**

The left hemisphere of each brain was processed in 4% paraformaldehyde (Sweet et al., 2009) and tissue blocks containing the superior frontal gyrus were sectioned coronally at 40  $\mu$ m on a cryostat and stored in antifreeze solution at -30°C until processing for immunohistochemistry.

#### **Immunohistochemistry**

Tissue sections were processed for fluorescent immunohistochemistry as described previously (Fish et al., 2008). Sections were incubated for 48 hrs in the following primary antibodies: GAD67 (mouse, 1:500, Millipore), GAD65 (rabbit, 1:1000, Millipore), and PV (goat, 1:1000, Swant, Bellinzona, Switzerland). Tissue sections were then incubated for 24 hrs with secondary antibodies (donkey) which were conjugated to Alexa 488 (1:500), 568 (1:500), or biotin (1:200; all from Invitrogen). For the biotin secondary, a tertiary incubation (24 hrs) with streptavidin 647 was performed. All sections were processed together in a single immunohistochemical run, and slides were coded so that subsequent steps were performed in a blinded fashion.

## **Microscopy**

Image stacks (512x512 pixels; 0.23  $\mu\text{m}$  z-step) were collected on an Olympus IX71 inverted microscope (Center Valley, PA) equipped with an Olympus DSU spinning disk confocal using a 60X 1.42 N.A. oil immersion objective and a Hamamatsu C9100 CCD camera (Bridgewater, NJ). SlideBook 4.2 software (Intelligent Imaging Innovations, Inc., Denver, CO) was used to control the microscope and for image processing. A total of 6-12 sites per section were imaged, with the number determined by the size of the region of interest. Running means were used to confirm that the number of sites per section was sufficient to adequately sample the region.

### **A.4 ANTIBODY SPECIFICITY**

All antibodies are commercially available and have had specificity demonstrated by multiple approaches per the manufacturers' data sheets. In addition, the specificity of each antibody was verified by Western blot in our laboratory (data not shown), with the exception of the PV antibody which has been verified by others (Kagi et al., 1987).

## **APPENDIX B**

### **COMPLETE SUBJECT TABLE**

**Table 4. Characteristics of human subjects used in this dissertation<sup>a</sup>**

Comparison Subjects									Subjects with Schizophrenia													
Pair	Case	Sex/ Race	Age	PMI <sup>d</sup>	Storage Time <sup>e</sup>	RIN	pH	Cause of Death	Case	DSM IV diagnosis	Sex/ Race	Age	PMI <sup>d</sup>	Storage Time <sup>e</sup>	RIN	pH	Cause of Death	Cannabis History	Nicotine ATOD	Antipsychotic ATOD	Antidepressant ATOD	Benzodiazepine/ VPA ATOD
1	592	M/B	41	22.1	159	9.0	6.7	ASCVD	533	Chronic undifferentiated schizophrenia	M/W	40	29.1	169	8.4	6.8	Accidental Asphyxiation	None	U	Y	N	N
2 <sup>a</sup>	567	F/W	46	15.0	163	8.9	6.7	Mitral Valve prolapse Homicide by gun shot	537	Schizoaffective disorder	F/W	37	14.5	168	8.6	6.7	Suicide by hanging	None	U	N	N	N
3 <sup>a</sup>	516	M/B	20	14.0	171	8.4	6.9		547	Schizoaffective disorder	M/B	27	16.5	167	7.4	7.0	Heat Stroke	None	U	Y	Y	Y
4 <sup>c</sup>	630	M/W	65	21.2	153	9.0	7.0		ASCVD	566	Chronic undifferentiated schizophrenia; AAR	M/W	63	18.3	164	8.0	6.8	ASCVD	None	Y	Y	Y
4 <sup>a,c</sup>	1322	M/W	62	16.5	37	8.6	6.8	ASCVD	--													
5	604	M/W	39	19.3	157	8.6	7.1	Hypoplastic coronary artery	581	Chronic paranoid schizophrenia; ADC; OAC	M/W	46	28.1	161	7.9	7.2	Accidental combined drug overdose	Use	U	Y	N	Y
6	546	F/W	37	23.5	167	8.6	6.7	ASCVD	587	Chronic undifferentiated schizophrenia; AAR	F/B	38	17.8	160	9.0	7.0	Myocardial hypertrophy	Use	Y	Y	N	Y
7	551	M/W	61	16.4	166	8.3	6.6	Cardiac Tamponade	625	Chronic disorganized schizophrenia; AAC	M/B	49	23.5	154	7.6	7.3	ASCVD	None	Y	Y	Y	N
8 <sup>a,b</sup>	685	M/W	56	14.5	146	8.1	6.6	Hypoplastic coronary artery	622	Chronic undifferentiated schizophrenia	M/W	58	18.9	154	7.4	6.8	Right MCA infarction	None	U	N	N	N
9 <sup>a</sup>	681	M/W	51	11.6	147	8.9	7.2	Hypertrophic cardiomyopathy	640	Chronic paranoid schizophrenia	M/W	49	5.2	152	8.4	6.9	Pulmonary embolism	None	U	Y	Y	N
10	806	M/W	57	24.0	125	7.8	6.9	Pulmonary embolism	665	Chronic paranoid schizophrenia; ADC	M/B	59	28.1	149	9.2	6.9	Intestinal hemorrhage	None	Y	Y	Y	N
11	822	M/B	28	25.3	123	8.5	7.0	ASCVD	787	Schizoaffective disorder; ODC	M/B	27	19.2	129	8.4	6.7	Suicide by gun shot	Dependence  Abuse in remission	N	Y	N	N
12 <sup>a</sup>	727	M/B	19	7.0	140	9.2	7.2	Trauma	829	Schizoaffective disorder; ADC; OAR	M/W	25	5.0	121	9.3	6.8	Suicide by salicylate overdose		Y	N	N	Y
13 <sup>a,b</sup>	871	M/W	28	16.5	112	8.5	7.1	Trauma	878	Disorganized schizophrenia; ADC	M/W	33	10.8	111	8.9	6.7	Myocardial fibrosis	Use	Y	Y	Y	Y
14 <sup>a</sup>	575	F/B	55	11.3	162	9.6	6.8	ASCVD	517	Disorganized schizophrenia; ADC	F/W	48	3.7	170	9.3	6.7	Intracerebral hemorrhage	None	Y	Y	N	N
15	700	M/W	42	26.1	144	8.7	7.0	ASCVD	539	Schizoaffective disorder; ADR	M/W	50	40.5	168	8.1	7.1	Suicide by combined drug overdose	None	U	Y	Y	Y
16	988	M/W	82	22.5	91	8.4	6.2	Trauma	621	Chronic undifferentiated schizophrenia	M/W	83	16.0	154	8.7	7.3	Accidental asphyxiation	None	U	N	N	N
17	686	F/W	52	22.6	146	8.5	7.0	ASCVD	656	Schizoaffective disorder; ADC	F/B	47	20.1	150	9.2	7.3	Suicide by gun shot	None	Y	Y	N	N
18	634	M/W	52	16.2	153	8.5	7.0	ASCVD	722	Chronic undifferentiated schizophrenia; ODR; OAR	M/B	45	9.1	140	9.2	6.7	Upper GI bleeding	Use	Y	Y	N	N
19 <sup>a,b</sup>	852	M/W	54	8.0	115	9.1	6.8	Cardiac tamponade	781	Schizoaffective disorder; ADR	M/B	52	8.0	130	7.7	6.7	Peritonitis	None	Y	Y	Y	N
20	987	F/W	65	21.5	91	9.1	6.8	ASCVD	802	Schizoaffective disorder; ADC; ODR	F/W	63	29.0	126	9.2	6.4	Right ventricular dysplasia	None	Y	Y	N	Y
21	818	F/W	67	24.0	124	8.4	7.1	Anaphylactic reaction	917	Chronic undifferentiated schizophrenia	F/W	71	23.8	104	7.0	6.8	ASCVD	None	Y	Y	N	N
22 <sup>a,b</sup>	857	M/W	48	16.6	114	8.9	6.7	ASCVD	930	Disorganized schizophrenia; ADR; OAR	M/W	47	15.3	100	8.2	6.2	ASCVD	Abuse	Y	Y	N	Y



Comparison Subjects									Subjects with Schizophrenia													
Pair	Case	Sex/ Race	Age	PMI <sup>d</sup>	Storage Time <sup>e</sup>	RIN	pH	Cause of Death	Case	DSM IV diagnosis	Sex/ Race	Age	PMI <sup>d</sup>	Storage Time <sup>e</sup>	RIN	pH	Cause of Death	Cannabis History	Nicotine ATOD	Antipsychotic ATOD	Antidepressant ATOD	Benzodiazepine/ VPA ATOD
23 <sup>a,b</sup>	739	M/W	40	15.8	139	8.4	6.9	ASCVD	933	Disorganized schizophrenia	M/W	44	8.3	100	8.1	5.9	Myocarditis	None	N	Y	Y	Y
24 <sup>a</sup>	1047	M/W	43	13.8	84	9.0	6.6	ASCVD	1209	Schizoaffective Disorder	M/W	35	9.1	64	8.7	6.5	Suicide by diphenhydramine overdose	None	N	Y	N	N
25	1086	M/W	51	24.2	78	8.1	6.8	ASCVD	10025	Disorganized Schizophrenia; OAR	M/B	52	27.1	57	7.8	6.7	ASCVD	Abuse in remission	Y	N	N	N
26 <sup>a</sup>	1092	F/B	40	16.6	77	8.0	6.8	Mitral Valve Prolapse	1178	Schizoaffective Disorder	F/B	37	18.9	69	8.4	6.1	Pulmonary embolism	None	Y	Y	N	Y
27	10005	M/W	42	23.5	65	7.4	6.7	Trauma	1256	Undifferentiated Schizophrenia	M/W	34	27.4	57	7.9	6.4	Suicide by hanging	None	N	Y	N	N
28	1336	M/W	65	18.4	42	8.0	6.8	Cardiac Tamponade	1173	Disorganized Schizophrenia; ADR	M/W	62	22.9	69	7.7	6.4	ASCVD	None	Y	Y	N	N
29 <sup>a</sup>	1122	M/W	55	15.4	74	7.9	6.7	Cardiac Tamponade	1105	Schizoaffective Disorder	M/W	53	7.9	76	8.9	6.2	ASCVD	None	Y	Y	N	N
30 <sup>a</sup>	1284	M/W	55	6.4	53	8.7	6.8	ASCVD	1188	Undifferentiated Schizophrenia; AAR; OAR	M/W	58	7.7	67	8.4	6.2	ASCVD	Abuse in remission	Y	Y	N	N
31	1191	M/B	59	19.4	66	8.4	6.2	ASCVD	1263	Undifferentiated Schizophrenia; ADR	M/W	62	22.7	56	8.5	7.1	Accidental asphyxiation	Use	Y	Y	Y	N
32	970	M/W	42	25.9	95	7.2	6.4	ASCVD	1222	Undifferentiated Schizophrenia; AAC	M/W	32	30.8	62	7.5	6.4	Suicide by combined drug overdose	Use	N	Y	Y	N
33	10003	M/W	49	21.2	66	8.4	6.5	Trauma	1088	Undifferentiated Schizophrenia; ADC; OAC	M/W	49	21.5	78	8.1	6.5	Accidental combined drug overdose	Abuse	Y	Y	Y	N
34	1247	F/W	58	22.7	58	8.4	6.4	ASCVD	1240	Undifferentiated Schizophrenia; ADR	F/B	50	22.9	59	7.7	6.3	ASCVD	None	Y	Y	N	N
35	1324	M/W	43	22.3	45	7.3	7	Aortic Dissection	10020	Paranoid Schizophrenia; AAC; OAC	M/W	38	28.8	58	7.4	6.6	Suicide by salicylate overdose	None	Y	Y	Y	Y
36 <sup>a</sup>	1099	F/W	24	9.1	76	8.6	6.5	Cardiomyopathy	10023	Disorganized Schizophrenia	F/B	25	20.1	57	7.4	6.7	Suicide by drowning	None	N	Y	N	Y
37 <sup>a</sup>	1307	M/B	32	4.8	48	7.6	6.7	ASCVD	10024	Paranoid Schizophrenia	M/B	37	6.0	57	7.5	6.1	ASCVD	None	N	N	N	N
38 <sup>a</sup>	1391	F/W	51	7.8	34.0	7.1	6.6	ASCVD	1189	Schizoaffective Disorder; AAR	F/W	47	14.4	67	8.3	6.4	Suicide by combined drug overdose	None	Y	Y	Y	Y
39	1282	F/W	39	24.5	53	7.5	6.8	ASCVD	1211	Schizoaffective Disorder	F/W	41	20.1	64	7.8	6.3	Sudden unexplained death	Use	Y	Y	Y	N
40 <sup>a</sup>	1159	M/W	51	16.7	70	7.6	6.5	ASCVD	1296	Undifferentiated Schizophrenia	M/W	48	7.8	50	7.3	6.5	Pneumonia	None	Y	Y	Y	N
41	1326	M/W	58	16.4	44	8.0	6.7	ASCVD	1314	Undifferentiated Schizophrenia	M/W	50	11.0	47	7.2	6.2	ASCVD	None	N	Y	Y	N
42	902	M/W	60	23.6	109	7.7	6.7	ASCVD	1361	Schizoaffective Disorder; ODC	M/W	63	23.2	39	7.7	6.4	Cardiomyopathy	Use	Y	Y	N	Y
	Mean	48.5	17.8	104.2	8.3	6.8						47.0	18.1	106.0	8.2	6.6		26Y/8N/ 8U	36Y/6N	17Y/25N	15Y/27N	
	SD	13.3	5.9	43.9	0.6	0.2						12.8	8.7	45.6	0.7	0.4						

Subjects with Schizophrenia (continued)						
Case	DSM IV diagnosis	Family History <sup>†</sup>	Age at Onset of Illness	History of Marriage	Hollingshead Two Factor ISP	Living Independently ATOD
533	Chronic undifferentiated schizophrenia	N	25	N	20	N
537	Schizoaffective disorder	N	29	Y	20	Y
547	Schizoaffective disorder	N	18	N	17	N
566	Chronic undifferentiated schizophrenia; AAR	N	43	Y	16	N
581	Chronic paranoid schizophrenia; ADC; OAC	Y	16	Y	19	Y
587	Chronic undifferentiated schizophrenia; AAR	N	18	N	17	N
625	Chronic disorganized schizophrenia; AAC	Y	35	Y	34	N
622	Chronic undifferentiated schizophrenia	N	42	N	27	N
640	Chronic paranoid schizophrenia	N	21	N	63	N
665	Chronic paranoid schizophrenia; ADC	Y	27	N	16	Y
787	Schizoaffective disorder; ODC	N	24	N	30	N
829	Schizoaffective disorder; ADC; OAR	Y	20	N	22	N
878	Disorganized schizophrenia; ADC	N	16	N	57	N
517	Disorganized schizophrenia; ADC	N	28	Y	53	N
539	Schizoaffective disorder; ADR	N	19	Y	45	Y
621	Chronic undifferentiated schizophrenia	Y	28	N	8	N
656	Schizoaffective disorder; ADC	Y	17	N	19	Y
722	Chronic undifferentiated schizophrenia; ODR: OAR	N	26	N	24	N
781	Schizoaffective disorder; ADR	N	37	Y	24	N
802	Schizoaffective disorder; ADC; ODR	N	20	N	42	Y
917	Chronic undifferentiated schizophrenia	Y	24	Y	19	N
930	Disorganized schizophrenia; ADR; OAR	N	19	N	17	N

Subjects with Schizophrenia (continued)						
Case	DSM IV diagnosis	Family History <sup>f</sup>	Age at Onset of Illness	History of Marriage	Hollingshead Two Factor ISP	Living Independently ATOD
933	Disorganized schizophrenia	N	22	N	27	N
1209	Schizoaffective Disorder	N	21	Y	35	Y
10025	Disorganized Schizophrenia; OAR	N	22	N	27	N
1178	Schizoaffective Disorder	N	26	N	40	N
1256	Undifferentiated Schizophrenia Disorganized	N	28	N	36	N
1173	Schizophrenia; ADR	N	29	Y	16	Y
1105	Schizoaffective Disorder Undifferentiated	N	48	N	24	N
1188	Schizophrenia; AAR: OAR Undifferentiated	Y	25	N	45	N
1263	Schizophrenia; ADR Undifferentiated	N	21	N	11	N
1222	Schizophrenia; AAC Undifferentiated	N	16	N	14	Y
1088	Schizophrenia; ADC: OAC Undifferentiated	N	25	Y	45	N
1240	Schizophrenia; ADR Paranoid	N	25	N	32	N
10020	Schizophrenia; AAC: OAC	N	18	N	27	N
10023	Disorganized Schizophrenia Paranoid	Y	15	N	17	Y
10024	Schizophrenia	N	20	Y	27	N
1189	Schizoaffective Disorder; AAR	N	43	N	66	Y
1211	Schizoaffective Disorder	N	29	Y	30	N
1296	Undifferentiated Schizophrenia	N	13	N	14	N
1314	Undifferentiated Schizophrenia	N	17	N	19	N
1361	Schizoaffective Disorder: ODC	N	16	N	17	N
		9Y/33N			13Y/29N	11Y/31N

<sup>a</sup>Pairs utilized in total protein study; <sup>b</sup>Pairs utilized in puncta study; <sup>c</sup>Subjects 630 and 1322 were used as the comparison subjects in pair 4 for qPCR and Western blotting studies, respectively; <sup>d</sup>PMI, postmortem interval (hours); <sup>e</sup>Storage time (months) at -80C; <sup>f</sup>First degree relative with schizophrenia; Other abbreviations: ASCVD, arteriosclerotic cardiovascular disease; MCA, middle coronary artery; Benzo, benzodiazepines; VPA, valproic acid; ATOD, at time of death; ADC, alcohol dependence, current ATOD; ADR, alcohol dependence, in remission ATOD; AAC, alcohol abuse, current ATOD; AAR, alcohol abuse, in remission ATOD; ODC, other substance dependence, current ATOD; ODR, other substance dependence, in remission ATOD; OAC, other substance abuse, current ATOD; OAR, other substance abuse, in remission ATOD; ISP, Index of Social Position.

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